Biomarkers of insulin resistance, oxidative stress, and nutrition and the brain

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Alzheimer's disease (AD) is a neurological disorder that has been linked with nutrition and lifestyle choices throughout the lifespan. Although there is currently no cure, recent research supports dietary changes as a preventative measure for AD. However, it is not clear which nutritional markers underlie the structural and functional changes in the brain that are seen in AD, and subsequently what aspect of the diet should be targeted in AD prevention and treatment. The projects described in this dissertation provide four nutritional biomarkers that are associated with AD pathology: the metabolism-regulating enzyme autotaxin, the insulin transporter and modifier insulin-like growth factor binding protein 2, the antioxidant superoxide dismutase 1, and the micronutrient involved in methylation of proteins and lipids as well as homocysteine metabolism, vitamin B12. These studies analyze neurological associations through region of interest approaches, voxel-wise analyses, and statistical analyses (i.e. linear mixed models, mediation models, logistic regression) with established cerebrospinal fluid (CSF) biomarkers of AD, cognitive test scores, and baseline and follow-up diagnoses. Our findings suggest that CSF autotaxin is detrimental to brain health and superoxide dismutase 1 is most likely beneficial. Insulin-like growth factor binding protein is suggested to be beneficial early in the AD trajectory, but detrimental as the disease progresses. Lastly, our results suggest that higher serum vitamin B12 may be indicative of worse AD outcomes in an aged population but contrastingly better cognitive associations in a young population. Future studies are needed to determine causation as well as the impact of nutritional modifications on these biomarkers and subsequent brain health.

CHAPTER 1. GENERAL INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that is the most common cause of dementia [(1)]. The impact of AD is wide-ranging, deteriorating memory, executive function, spatial orientation, and activities of daily living [(2)]. In 2017, there were approximately 3.65 million individuals with AD, and this is estimated to expand to 9.3 million by the year 2060 [(3)]. While AD affects less than 1% of individuals under the age of 60, the prevalence rises to 40% in the population of individuals over the age of 85 [(4)]. AD has been associated with the accumulation of tau tangles and amyloid plaques in the brain, but the underlying causes of these protein changes in the brain is yet to be determined [(1)].

Nutrition and is thought to be involved as high dietary intake of antioxidants and omega-3 fatty acids and moderate alcohol consumption are linked with decreased risk for AD [(4)]. On the other hand, obesity in the midlife is associated with a 2.4 increase in odds for developing AD [(5)]. Further, midlife diabetes is associated with a 1.42 increased odds ratio for AD [(6)]. Compellingly, mice that were fed components that make up the typical western diet (high fat and saturated fat, elevated simple carbohydrates and low omega-3 and omega-6 fatty acids) led to both a loss of hippocampal neuronal cells and formation of reactive astrocytes in the hippocampus, which is greatly affected by AD [(7)]. The potential mechanisms behind these relationships, which will be discussed further in this dissertation, are thought to involve inflammation, oxidative stress, and dysregulated metabolism within the brain.

The determination of clinical biomarkers of AD, or its precursor mild cognitive impairment (MCI), can improve diagnostic accuracy and provide potential new drug targets

for improving symptoms, slowing progression, and bring the scientific community closer to more effective treatments and a cure [8]. Besides tau and amyloid, current prominent biomarkers for AD include neurofilament light protein, neuron-specific enolase, visinin-like protein 1, heart fatty acid binding protein, and the glial activation marker YKL-40, however a current issue is lack of autopsy confirmation of biomarkers [9]. Because of the evidence surrounding the role of nutrition in AD, nutrition-related biomarkers may track neurodegeneration and also provide a modifiable target through dietary changes.

Dissertation Organization

This dissertation is made up of eight chapters with a general introduction, a literature review, five manuscript-style chapters, and a general conclusion. The first manuscript chapter titled "Autotaxin is Related to Metabolic Dysfunction and Predicts Alzheimer's Disease Outcomes" explores the role of the insulin-resistance-related biomarker, autotaxin, in AD and was published in the *Journal of Alzheimer's Disease*. Continuing the theme of insulin resistance, the next chapter is titled "Peripheral versus Central Index of Metabolic Dysfunction and Associations with Clinical and Pathological Outcomes in Alzheimer's Disease" which assesses insulin-like growth factor binding protein 2 (IGFBP-2) as a marker of changes in grey matter, cerebral glucose metabolism, and cognitive functions. This manuscript was also published in the *Journal of Alzheimer's Disease.* The third manuscript chapter centers on reactive oxygen species and how the antioxidant superoxide dismutase (SOD) may track AD-related changes as well as its relationship with tau. This manuscript is titled "Is CSF SOD1 a Biomarker of Tau but not Amyloid Induced Neurodegeneration in Alzheimer's Disease?" and is currently in press with

the journal *Antioxidants & Redox Signaling*. Transitioning to vitamin B12, the fourth manuscript chapter examines the role of vitamin B12 in grey matter and brain metabolism outcomes and is titled "Serum Vitamin B12, and Related Cubilin Genotypes, Predict Neural Outcomes across the AD Spectrum" and is currently in revision with *The British Journal of Nutrition*. The last manuscript-style chapter consists of preliminary results regarding vitamin B12 and cognition from the study Obesity, Signal Imaging, and Reactions at Iowa State (OSIRIS) and is titled "Serum Vitamin B12 is Related to Improved Executive Function in Young Adults". This manuscript will be prepared for submission to a scientific journal after further data from the study becomes available for additional analyses.

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CHAPTER 2. LITERATURE REVIEW

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive, degenerative disorder characterized by loss of brain cells, or neurons, in individuals typically over the age of 65 [(1)]. This leads to the loss of memory, disorientation, psychiatric symptoms, decline of activities of daily living, and confusion [(2)]. Small changes are thought to begin in the brain 20 years before a patient may notice symptoms [(1)]. Alterations in two types of proteins are thought to play a role in the pathogenesis of AD: beta amyloid plaques and tau tangles [(3)]. These plaques and tangles can prevent communication between neurons, as well as deteriorate their structure and function[(4)]. Approximately one-third of older adults show brain changes related to AD without any presenting symptoms, suggesting that there is still a disconnect between diagnostic criteria and symptoms and a need for further biomarkers of the disease [(5)]. An absolute diagnosis of AD can only be made at autopsy, however criteria are set for probable clinical diagnoses using neuropsychological examinations, reports from the patient and an informant about activities of daily living, and a complete history of symptoms [(4)]. Currently, AD is clinically diagnosed in the United States using the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [(4)]. Mild Cognitive Impairment, or MCI, is the precursor to AD. Patients with MCI show lower scores on one or greater cognitive domain, when assessed objectively [(6)]. However, these patients are still able to complete basic activities of daily living, such as dressing, bathing, or hygiene [(7)].

Areas of the brain that are most susceptible to AD-related degeneration include: the medial temporal lobe, the lateral temporal and parietal areas, the cingulate cortex, and the frontal lobe [8]. The medial temporal lobe is made up of the hippocampus, the entorhinal cortex, and the parahippocampal gyrus [9]. These areas are important for episodic memory (events), associative memory (associating two memories together), semantic memory (general facts), and spatial memory [9]. The lateral temporal cortex plays important roles in auditory memory, reading and naming visual stimuli [10]. The lateral parietal cortex is also thought to be involved in memory retrieval [11] and the cingulate cortex is implicated in emotion, executive function, spatial orientation, and also has connections to the parahippocampal gyrus and is thought to be involved in memory [12]. Lastly, the frontal lobe is engaged in attention, executive function, decision making, language, personality, affect, and mood [13].

Cognitively, patients with AD are known to experience numerous deficits. Autobiographical memory degradation is a hallmark symptom of AD, where patients are first unable to form new memories, and then unable to remember memories from the past [14]. Executive function, which is involved in planning, controlling behavior, and inhibition, is diminished in AD [15]. Further, spatial disorientation is common in patients with AD and individuals with MCI often have difficulty with navigation [16]. Depression, irritability, and other mood disturbances are also common [17].

AD is currently the 6th leading cause of death in the United States. It is estimated that there are currently 5.8 million Americans living with AD [1]. Medical and other related expenditures for Alzheimer's disease were responsible for a cost of $290 billion in the

United States in 2017 [1]. Close to two-thirds of individuals with Alzheimer's disease are women, and African-Americans are twice as likely to be diagnosed with Alzheimer's disease as Caucasian individuals[1].

Apolipoprotein ε4 or APOE ε4 is considered a genetic risk factor that contributes to AD. Apolipoproteins serve to transport cholesterol and phospholipids [18]. Three isoforms of APOE exist: APOE ε2, APOE ε3 and APOE ε4, stemming from amino acid changes at residues 112 and 158 of the APOE gene [19]. APOE ε4 is less stable than the more common APOE ε3, and more likely to be degraded by astrocytes, which maintain the blood brain barrier and act as neuronal-repair cells [18]. Additionally, APOE ε4 has a higher affinity for binding to VLDL in the blood, compared to APOE ε2 and APOE ε3, leading to an increase in LDL in the blood [19]. Importantly, apolipoproteins are postulated to be involved in beta amyloid clearance from the brain, so it can be asserted that individuals who have at least one copy of APOE ε4 are less able to efficiently clear beta amyloid plaques [18]. It is currently estimated that one copy of the APOE ε4 allele is associated with a 3 times increased risk for AD, while possessing two copies is associated with a 12-fold increased risk for AD [20]. Conversely, the APOE ε2 allele is considered protective against developing AD, where having at least one ε2 allele led to a 0.08 odds ratio for AD compared to having at least one ε3 allele with an odds ratio of 0.16 [21]. Additional gene mutations are associated with the development of AD including: amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2), clusterin (CLU), Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) and Phosphatidylinositol-Binding Clathrin Assembly Protein (PICALM) [20; 22].

Many theories have been proposed in determining the root cause of AD. Although it is not known conclusively why amyloid amasses in those with AD, it is thought to be related to dysfunction of the APOE gene and cholesterol metabolism [23]. Cholesterol-rich domains in the lipid bilayer of cell membranes are thought to be involved in the production of amyloid precursor protein; both serum total cholesterol and LDL cholesterol have been correlated with beta amyloid plaques in the brain [24; 25]. As amyloid plaques accumulate, an inflammatory cascade is put in motion, where the activated glia cells that surround neurons, astrocytes, begin releasing cytokines in an attempt to control the offending plaques [22]. In excess, these cytokines can lead to chronic neuroinflammation and neurodegeneration [26]. In addition, glial fibrillary acidic protein is released from astroctyes which corresponds with the uptake of the excitatory neurotransmitter glutamate, thus sequestering glutamate from the neurons to prevent hyperexcitation of the neurons and apoptosis [26; 27]. As glutamate is taken up into the astrocytes, glutamate is converted to glutamine, which has been found in higher levels of AD patients [26; 28; 29]. These astrocytes begin to hypertrophy and release higher amounts of the inhibitory neurotransmitter, GABA [30]. It can be hypothesized that these changes lead to the dysregulation of neurotransmitters that is seen in AD [22]. Another hypothesis is that cholinergic neurons, neurons that use acetylcholine as signaling molecules, dysfunction [31]. Neuroinflammation has been shown to lead to a decrease in nerve growth factor, which binds to receptors on cholinergic neurons and is thought to be necessary for their survival, especially in the forebrain [31]. Transitioning to tau, tau is a microtubule protein that is found in neurons and is regulated through post-translational phosphorylation [32]. Mutations in the gene that

encodes for tau, MAPT, have been linked with neurodegeneration [23]. Abnormal phosphorylation of tau can lead to aggregations of the protein which can in-turn disrupt the transport of neuron to neuron signals via axons [23].

Despite the increased research focus and federal funding, there is currently no cure for AD, and clinical treatments focus on symptom management [33]. Drugs that target amyloid production by β- and γ- secretase inhibitors failed to meet effectiveness standards or were deemed unsafe for human consumption [33]. Immunotherapy has also been investigated as a potential target for amyloid proteins but a major clinical trial was discontinued due to 6% of subjects in the treatment group developing meningoencephaltis [34]. The drug memantine is a NMDA receptor agonist which is thought to decrease sustained blockage of NMDA receptors that is seen in AD [35]. Memantine is currently approved for the treatment of AD, however only some studies have found utility of the drug, in the form of small improvements in memory or activities of daily living [35]. Cholinesterase inhibitors are also prescribed to individuals with AD, which decrease the breakdown of acetylcholine in the synaptic cleft, thereby increasing neuronal communication, which leads to small increases in global cognition and activities of daily living [36]. Interestingly, there is potential for rheumatoid arthritis drugs that block the production of the protein tumor necrosis factor alpha (TNFα) as an AD prevention or treatment. Though the drug is too large to cross the blood brain barrier, its use has been associated with decreased risk for AD, highlighting the importance of controlling peripheral inflammation [37].

Obesity and Insulin Resistance

Obesity is linked with heart disease, diabetes, cancer, joint ailments, and many other chronic diseases [38]. According to the Centers for Disease Control and Prevention, more than one third of adults in the United States have obesity [39]. Additionally, it is estimated that obesity was responsible for $147 billion dollars in medical costs in the U.S. in 2008 [39]. Some minority groups are disproportionately affected by obesity, with Hispanic having the highest rates (47.0%) followed by non-Hispanic blacks (46.8%) [39].

Strong correlations have been shown between obesity and insulin resistance (IR), which is defined as the progressive inability for insulin to bind to its receptor [40]. This is thought to be due to increases in inflammatory factors, as well as the release of non-esterified fatty acids (NEFAs) from adipose tissue, creating a competition with glucose for energy substrates for cells and a down-regulation of certain glycolysis enzymes [41]. The raised levels of circulating glucose and NEFAs are thought to cause the pancreatic beta cells to increase in mass and secrete higher levels of insulin, leading to an increased level of insulin required for cells to uptake glucose [41]. In the Nurses' Health Study cohort, BMI was correlated with a diagnosis of diabetes, and women with a BMI of 25-26.9 had a five times increased risk for diabetes compared to women with a BMI of under 22 [42]. In a large clinical trial with individuals with prediabetes and a BMI of 24 or greater, individuals who received an intensive weight-loss lifestyle intervention lost an average of 5.6 kg and had a 58% lower risk of being diagnosed with diabetes compared to a placebo group and a 27% reduced risk of diabetes compared to a group on metformin therapy [43]. Even individuals

with obesity that were considered metabolically healthy had four times the risk of being diagnosed with type 2 diabetes compared to healthy lean individuals [44].

It has been estimated that by the year 2030, there will be 439 million individuals world-wide with diabetes, which is a 54% increase from the year 2010 [45]. Type 1 diabetes is a genetic auto-immune disease where beta cells of the pancreas are attacked, and it affects about 5-10% of those with diabetes. About 90% of individuals with diabetes have type 2 diabetes, which is related to IR [46]. Risk factors for type 2 diabetes include: diets low in fiber, high in glycemic index foods, high in trans-fat and saturated fat; low physical activity; smoking; elevated levels of alcohol intake; and increased levels of total adiposity [47].

Many studies have linked obesity and IR with wide-ranging inflammation [48; 49]. Human capillary endothelial cells treated with adipocyte cell medium showed increased adhesion of monocytes (a type of white blood cell which can indicate inflammation) compared to endothelial cells grown in a control medium [50]. Additionally, these authors found a positive correlation between the number of macrophages that were present in human adipose tissue and BMI [50]. Other studies have shown increased markers of inflammation in adipose tissue from obese individuals, such as tumor necrosis factor- alpha, interleukin-6, and monocyte chemotactic protein-1 [51]. Further, adipose tissue sampled from lean and obese individuals showed significantly higher expression of Cd68, a low-density lipoprotein that is expressed on macrophages and monocytes, in adipose tissue sampled from obese participants, compared to lean [51]. Lastly, the same study showed that

there was a positive correlation between average adipocyte cross-sectional area and Cd68 levels, indicating that enlarged adipocytes are inflammatory to the body [51].

Obesity and the Brain

Obesity is associated with decreased grey matter and metabolic function in the brain [52; 53]. Further, obesity is associated with decreased blood flow to the brain, which correlates with brain tissue degeneration [54]. Rats fed a high fat diet for 16 weeks showed higher levels of the inflammatory marker TNFα in the hypothalamus compared to rats fed a control diet [55]. In the observational UK Biobank study, total body fat was associated with less grey matter in the thalamus, caudate nucleus, putamen, globus pallidus, hippocampus, amygdala, and nucleus accumbens for male participants but only related to less grey matter in the globus pallidus in women, which the authors postulated may have been due to a protective effect of estrogen [56]. In contrast, higher total body fat was linked to increased white mater integrity in both male and female participants [56].

However, the relationship between obesity and brain degeneration may be related to obesity comorbidities. One study showed that BMI was associated with cognitive tests related to attention and verbal fluency, but memory, processing speed, and executive function were only correlated with obesity-related comorbidities such as diabetes, CPAP usage, and apnea [57].

As an alternative or concurrent theory, the inflammation associated with obesity may determine neuronal degeneration. Obesity is associated with an increased production of cytokines, which have the ability to cross the blood brain barrier [58]. High levels of C-reactive protein, an inflammation marker, have been associated with increased white

matter damage in individuals free of dementia [59]. In participants ranging from age 35 to 85 years of age from the Framingham Heart Study, there was an overall negative effect of nine blood-based inflammatory markers on total brain volume adjusted for head size [60]. Further, for middle-aged to aged participants from the Rotterdam Study, participants in the highest quartile for baseline plasma inflammatory proteins compared to the lowest quartile had a 2.92 increased odds ratio for developing dementia [61]. Providing a potential cause and effect, a high fat diet induced model of obesity in rodents led to an increased level of $CD68^+$ activated microglia in the hippocampus of aged mice compared to mice fed a control diet [48].

Insulin Resistance and the Brain

Normal aging results in progressive IR, due to increased fat mass and decreased lean muscle mass [62]. Insulin resistance in cognitively normal participants in late middle age led to lower global glucose metabolism in the brain as well as decreased immediate and delayed memory [52]. Additionally, higher IR was associated with decreased cerebral glucose metabolic rate and worse memory scores in cognitively normal adults with pre-diabetes or type 2 diabetes [63]. Further, diabetes was significantly associated with worse cognitive performance and attention deficit in young adults without dementia [64]. Last, a lower insulin sensitivity index in rhesus monkeys was associated with less medial temporal lobe (MTL) and prefrontal cortex (PFC) gray matter, which mediated worse executive function performance [65].

Alzheimer's disease (AD) has been linked with dysregulation of the insulin-signaling pathway [66]. This manifests in structural and functional deficits of the brain. The Mayo

Clinic Study of Aging showed that in a cohort of late-aged adults, midlife diabetes was associated with decreased executive function scores, decreased global cognition, and a higher odds ratio for being diagnosed with MCI [7]. Individuals with type 2 diabetes in the Alzheimer's Disease Neuroimaging Initiative (ADNI) showed decreased [18F]-Fluorodeoxyglucose (FDG)-PET uptake on a whole-brain level compared to individuals without a diagnosis of type 2 diabetes. In addition, in a subset of individuals with MCI, a concurrent diagnosis of diabetes was related to less FDG uptake in the frontal lobe, sensory motor cortex, and striatum compared to individuals who had MCI but no type 2 diabetes diagnosis [67].

Several studies have made convincing arguments that glycemic control determines these pathological changes. In the Mayo Clinic Study of Aging, a diagnosis of diabetes was associated with incidence of subcortical infarctions, and individuals with diabetes that were receiving pharmacologic treatment displayed lower levels of subcortical infarctions compared to participants with diabetes that were not receiving treatment, suggesting that uncontrolled hyperglycemia and damage to the brain vasculature may be linked [68]. To further this notion, use of the anti-diabetes medication pioglitazone, which regulates the control of insulin via gene transcription, in individuals with non-insulin dependent diabetes was matched with decreased risk for dementia compared to individuals who did not take the drug [69]. Interestingly, hemoglobin A1C levels mediated the relationship between duration of type 2 diabetes and worse executive function scores, where individuals with higher hemoglobin AIC had worse executive function scores [70].

Interventions aimed at improving insulin sensitivity may slow the cognitive decline seen in MCI. Moderate calorie restriction has been shown to increase insulin sensitivity and decrease hemoglobin A1C and fasting blood glucose [71]. Anson and colleagues found that on average, mice that were only given access to food every other day and mice that were given ad libitum food access consumed the same amount of food over a 48-hour period. However, the researchers found that the intermittent CR mice had significantly lower fasting serum insulin and glucose levels, compared to ad libitum food access mice (100 mg/dL glucose vs. 150 mg/dL and 1,100 pg/mL insulin vs. 3,400 pg/mL) [72]. The authors postulated that the mice were better able to metabolically adapt to smaller periods of high intake than constant intake. Further, rats that were fed a CR diet showed decreased mitochondria damage as they aged, compared to control fed rats [73]. A 500-calorie deficit a day or minimum 1200 kcal/day diet in subjects with MCI and obesity led to weight loss, with higher weight loss leading to improved verbal memory, language, and executive function [74]. In a cohort of bariatric surgery patients, as HOMA-IR levels decreased following the surgery, cognition scores improved [75]. In addition, daily intranasal (delivered directly to the central nervous system) insulin therapy for four months in individuals with MCI or AD led to significantly improved delayed recall and caregiver rated activities of daily living [76]. Aerobic exercise is capable of increasing the effectiveness of the insulin-dependent GLUT4 glucose transporter [77]. The Alzheimer's Disease Exercise Program Trial showed that individuals with MCI who were randomized to engage in 150 minutes of aerobic physical activity per week showed an increase in functional abilities but no statistical differences from control in the areas of memory and executive function [78].

The Insulin Signaling System and the Brain

The insulin signaling system is made up of insulin, insulin-like growth factors (IGF) 1 and 2, insulin-like growth factor binding proteins (IGFBP) and insulin receptors [79]. IGF system-wide functions include: DNA synthesis, regulating cell proliferation, controlling cell death, and uptake of glucose and amino acids into the cells [80; 81]. IR and T2D are preceded by dysregulation of insulin-like growth factor-1 (IGF-1) [82].

Insulin crosses the blood brain barrier and can increase the transport of certain amino acids and the satiety hormone leptin across the blood brain barrier [83]. Even though the brain was assumed to be insulin-independent due to its superseding utilization of glucose, insulin receptors are located in the neurons and glia, and are now known to be important for brain glucose metabolism [84]. The hypothalamus, a region important for body homeostasis, contains GLUT 4 glucose transporters, which are insulin-dependent. In addition, the hippocampus and cerebellum also contain GLUT 4, indicating their responsiveness to insulin [83]. However, the role of insulin in the brain may be for purposes outside of glucose uptake, as all areas of the brain contain GLUT 1 and GLUT 3, which are insulin-independent [85]. To support this, insulin binding has been associated with regulatory roles in the brain such as neurotransmitter uptake and degradation and altering the sensitivity of post-synaptic neurons [86].

Insulin receptor dysregulation is thought to play a role in AD. When beta amyloid was introduced to hippocampal neurons, insulin receptors redistributed on the neurons, where the neurons no longer showed insulin receptors on the dendrites of the neurons, indicating that amyloid formation and insulin receptor dysfunction may be bidirectional [87].

Post-mortem brain slices from individuals with frontotemporal dementia, which is also marked by grey matter loss and accumulation of tau, showed decreased immunoreactivity to IGF-1 in the frontal lobe compared to control brain slices [88]. With all of these points considered, it is of great interest to determine if pharmaceutical or diet/lifestyle interventions may exist to maintain proper insulin signaling in the brain especially in the setting of system-wise insulin resistance.

Reactive Oxygen Species

Reactive oxygen species are reactive superoxide anions produced during normal function of the electron transport chain in the mitochondria, as well as a result of ionizing radiation, activation of neutrophils and macrophages, lipid peroxidation, air pollutants, and glycation glycoxidation products [89; 90]. These reactive oxygen species can build up over time and cause damage to protein, lipids, and nucleic acids [90]. Additionally, free radicals can oxidize low density lipoprotein (LDL) cholesterol, which can lead to atherosclerosis of the arteries and endothelial dysfunction [91]. On the other hand, reactive oxygen species are beneficial and necessary in small amounts, by way of signal transduction, regulation of biological processes, and defense against pathogens [92].

Nutrition and Reactive Oxygen Species

Nutrition plays an important role in reactive oxygen species quenching, as vitamin C, vitamin E, glutathione and beta carotene are all able to be oxidized in place of physiological substrates [93]. Additionally, the enzymes superoxide dismutase (SOD), glutathione peroxidase, and catalase are produced by the body in order to control free radical concentration and limit damage [93]. These enzymes can also be influenced by diet. One

study showed a positive association between leafy green and cruciferous vegetable intake and erythrocyte SOD activity and a negative association between flour and grain products and erythrocyte SOD activity [94]. The authors also found a positive association between breakfast cereal consumption and glutathione peroxidase concentrations and a negative association between milk, vegetable dishes, beans and lentils and glutathione peroxidase [94]. Additionally, a study which dosed 13 aged men with 0.1 g/kg body weight garlic daily for one month showed a statistically significant increase in erythrocyte SOD compared to baseline [95].

Antioxidant System, Aging, and AD

Over time, the antioxidant system in the body becomes less efficient, which is evidenced by the production of less active antioxidant enzymes [90]. In addition, premature aging has been associated with increased oxidative damage, especially in mitochondria protein [96]. A statistically-significant increased level of oxidative damage to proteins has been shown in fibroblast cells from donors over the age of 60 compared to individuals less than 60 years of age [97].

Reactive oxygen species are thought to play a role in the onset and progression of AD. Increased levels of isoprostanes, markers of lipid peroxidation, were shown to be significantly higher in the urine, plasma and CSF of AD and MCI participants, compared to controls [98]. Additionally, a strong link between beta amyloid plaques and oxidative damage has been shown [99]. Further, addition of beta amyloid to rat embryonic hippocampal cell cultures causes an increase in ornithine decarboxylase, indicative of free radical damage, and cell death, but this is mitigated by the addition vitamin E to those

cultures [100]. It is unclear if the oxidative damage seen in AD is a result of the disease process or causative of neurological decline.

Despite the evidence of oxidative damage in AD, supplementation with single antioxidants has not been shown to be beneficial with respect to brain health [101]. However, when antioxidant intake was assessed based on dietary recall, there was an inverse relationship between vitamin E intake and risk for dementia at a mean follow-up time of 9.6 years [102]. Additionally, consumption of omega-3 rich oils and daily consumption of fruits and vegetables was related to a decreased risk for dementia at four-year follow-up in individuals 65 years of age or older, indicating a protective effect of antioxidant-rich foods [103].

Superoxide Dismutase (SOD)

SOD is an antioxidant enzyme that catalyzes the oxidation-reduction reaction of the superoxide anion ($O_2^{\bullet -}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2) [104]. SOD consists of three isoenzymes: copper/zinc SOD, or SOD1, localized predominantly to the cytosol; manganese SOD, or SOD2, within mitochondria; and extracellular SOD, or SOD3. Altered levels of SOD can cause excess ROS in cells, leading to altered intracellular and mitochondrial metabolism, as well as DNA and vascular damage [105; 106; 107; 108].

Interestingly, synthetic SOD injected into mice shows that increased SOD levels may reverse cognitive dysfunction induced by oxidative stress and aging [109]. However, SOD1 overexpression in the hippocampus of middle-aged rats silences N-methyl-D-aspartate receptor channel activity, a molecular mechanism of synaptic plasticity underlying learning, which coincides with difficulty in learning a cue discrimination task and oxidative stress in

hippocampal tissue [110]. These effects seem specific to SOD1, because overexpression of SOD2 has no effects on synaptic plasticity [111], and partial null mutations of SOD2 lead predominantly to increased cerebrovascular damage but less Aβ in amyloid precursor protein (APP) mice [112]. These results suggest that SOD1 specifically is integral to synaptic plasticity and memory formation.

SOD and AD

By extension, SOD1 has been examined in AD for its role in preserving neural integrity and memory performance. SOD affects mitochondrial integrity and free radical levels [113], and these free radicals are in turn linked to beta-amyloid (Aβ) associated neurodegenerative etiopathogenesis [114]. Overexpression of APP and SOD1 in transgenic mice shows pronounced atrophy in hippocampus and cortical regions, whereas SOD1 alone induces cortical atrophy, suggesting AD-like changes. It is not surprising that increased SOD1 levels are seen in the pyramidal neurons of AD patients compared to controls [115]. When examining single nucleotide polymorphisms (SNPs) among SOD1 and SOD3 genes for AD patients and controls, the only "hit" found was for SOD1 rs2070424 minor G allele carriers who showed a 50% reduction in AD risk [116]. For hippocampal cells of AD patients and related transgenic models, SOD1 overexpression downregulates Aβ-induced reductions of the non- versus oxidized glutathione ratio, a marker of oxidative stress [117], whereas SOD1-deficient Tg2576 mice show increased Aβ plaque formation, neuroinflammation, and memory decline [118]. Further, co-expression of SOD1 in APP transgenic mice blocks endothelial dysfunction that gives rise to hypoperfusion often seen in AD [119]. Finally, lifestyle interventions may affect SOD1 and neurodegenerative processes, where exercise

among transgenic tauopathy mice induces SOD1 expression that coincides with markedly lower phosphorylated tau levels in CA3 of hippocampus [120].

Despite the critical role of SOD1 in maintaining synaptic plasticity and memory, as well as its dysregulation in AD and AD transgenic models, SOD1 levels have not been fully studied as a biomarker of AD onset and progression. In a cohort of Malayasian older adults, participants with cognitive decline had significantly lower levels of plasma SOD compared to healthy controls [121]. Previously, baseline SOD-1 among Down's syndrome patients, who almost universally develop AD neuropathology, has not predicted cross-sectional memory performance [122] but does strongly track longitudinal decline [123].

Vitamin B12

Vitamin B12, also known as cobalamin, is a water-soluble vitamin that is necessary for red blood cell formation and DNA synthesis and contributes to the production of myelin the central nervous system [124]. The vast majority of vitamin B12-rich foods are of animal origin, causing difficulties for vegans and even lacto-ovo-vegetarians to meet their vitamin B12 needs [125]. The few plant sources of vitamin B12 include: tempeh (due to fermentation), certain types of mushrooms, tea leaves, and edible algae [126]. Vitamin B12 cannot be produced my humans or other animals, as only some bacteria and archaea have the ability to produce the micronutrient [127]. Although vitamin B12 is produced by gut microbiota, it cannot be absorbed by the host as its production is past the absorption site in the ileum [128]. See **Table 2.2** for a listing of common food sources of vitamin B12.

When vitamin B12 is consumed, it binds to transcobalamin I, which resides in the saliva [125]. Once the bound vitamin B12 reaches the stomach, stomach acid separates it

from transcobalamin I, where it binds to intrinsic factor, which is produced in the parietal cells of the stomach. The bound intrinsic factor and vitamin B12 travel through the small intestine to the ileum, where vitamin B12 is absorbed via the protein cubilin into the ileum enterocyte [125]. Vitamin B12 is transported through the blood via transcobalamin II or via another carrier, haptocorrin to target tissues [125]. Vitamin B12 travels to the kidney for control of the amount of circulating vitamin B12 via the protein megalin and to the liver to act as a cofactor in vitamin B12-dependent reactions [129].

Table 2.1 Recommended Dietary Allowances for Vitamin B12

Age	Male	Female	Pregnancy	Lactation
0-6 months*	0.4 mcg	0.4 mcg		
7-12 months*	0.5 mcg	0.5 mcg		
1-3 years	0.9 mcg	0.9 mcg		
4-8 years	1.2 mcg	1.2 mcg		
9-13 years	1.8 mcg	1.8 mcg		
14+ years	2.4 mcg	2.4 mcg	2.6 mcg	2.8 mcg

Adapted from [124]

Table 2.2 Food Sources of Vitamin B12

Food	Mcg per serving	Percent DV
Clams, cooked, 3 oz	84.1	1,402%
Nutritional yeast, fortified with 100% DV B12, 1 serving	6.0	100%
Salmon, sockeye, cooked, 3 oz	4.8	80%
Breakfast cereals, fortified with 25% DV B12, 1 serving	1.5	25%
Beef, top sirloin, broiled, 3 oz	1.4	23%
Milk, low-fat, 1 cup	1.2	18%
Egg, whole, hard boiled, 1 large	0.6	10%
Chicken breast, roasted 3 oz	0.3	5%

*Based on a daily value of 6.0 mcg

Adapted from [124]

Vitamin B12 Deficiency

Vitamin B12 deficiency is defined as serum vitamin B12 values below approximately 170-250 pg/mL [124]. However, methylmalonic acid levels above 0.4 μmol/L may be a more sensitive indicator, as vitamin B12 is required for methylmalonic acid to be converted to succinyl-CoA for metabolism of odd-chain fatty acids [124]. Symptoms of vitamin B12 deficiency include weakness, fatigue, trouble concentrating, constipation and depression [124]. Vitamin B12 deficiency causes megaloblastic anemia and is more common in the aged population due to decreased intrinsic factor production [130]. In a more rare but serious manner, pernicious anemia can lead to serious vitamin B12 deficiency due to a deficiency of intrinsic factor [131]. B12 deficiency is particularly prevalent in India and parts of Africa due to lower consumption of animal products [131]. Additionally, gastric bypass surgeries lead to vitamin B12 malabsorption due to loss of much of the stomach and subsequent parietal cells. Intramuscular or sublingual vitamin B12 is routinely prescribed to gastric bypass patients in order to avoid vitamin b12 deficiency [132]. Chronic use of proton pump inhibitors for management of acid reflux has been linked with vitamin B12 deficiency, presumably due to the suppression of stomach acid, which is necessary to release the consumed vitamin B12 from its food matrix [133]. Additionally, use of the diabetes medication metformin can significantly decrease serum vitamin B12 levels, most likely due to changes in calcium cations, which are necessary for intrinsic factor to bind to vitamin B12 for absorption into the ileum enterocyte [134].

One Carbon Metabolism

In the methionine homocysteine cycle, methionine can be converted to s-adenosyl-methionine or SAM [135]. SAM has the ability to methylate DNA, RNA, and proteins, which can regulate gene expression [135]. After SAM donates a methyl group, s-adenosyl-homocysteine is generated, which is then converted to adenine and homocysteine [136]. Homocysteine can then be converted to cystathione with the addition of serine via the transulfuration pathway or regenerated to methionine via methionine synthase, which requires vitamin B12 as a cofactor and 5-methyltetrahydrofolate as a methyl group donor [137]. An additional remethylation pathway exists, where choline is oxidized to betaine and donates a methyl group, regenerating methionine [137].

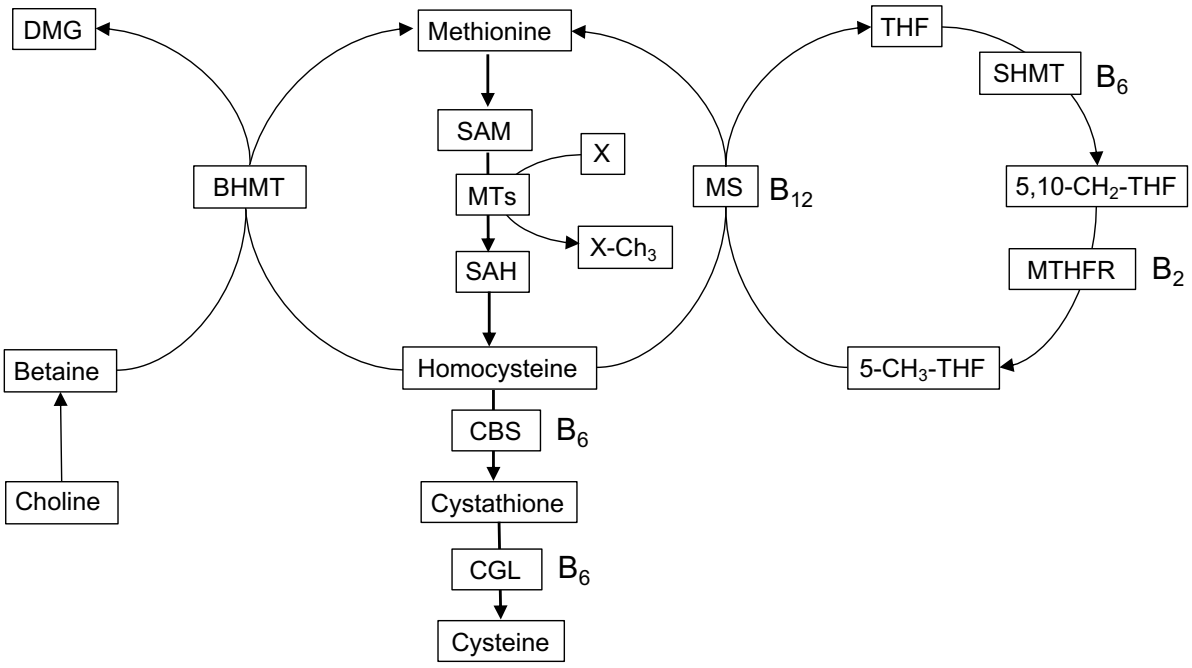

Figure 2.1 Homocysteine-Methionine Cycle

Adapted from [137]

DMG= dimethylglycine; SAM= S-adenosylmethionine; SAH= S-adenosylhomocysteine; MS= methionine synthase; THF= tetrahydrofolate; CBS=cystathionine β-synthase; CGL= cystathionine γ-lyase; BHMT=betaine-homocysteine *S*-methyltransferase; MTs= methyltransferases; MTHFR=5,10-methylene-THF reductase; SHMT=serine hydroxymethyltransferase

Vitamin B12 and Cognition

Because of vitamin B12's role in the nervous system, there has long been interest in how vitamin B12 may affect cognition. Compared to the older population, there has been limited research in the younger adult population with regard to vitamin B12 and cognitive outcomes. Qin and colleagues assessed vitamin B12 in a cohort of young adult females three times over 25 years. At the end of 25 years, cognitive testing showed that individuals who consumed in the top quintile of vitamin B12 compared to their peers showed significantly better scores in the digit symbol substitution test, which assesses executive function [(138)]. By way of other positive associations, a cohort of young women showed a positive correlation between usual dietary vitamin B12 and vitamin B6 intake and recognition memory in the Rey Auditory Verbal Learning Test [(139)].

Studies reporting the impact of vitamin B12 on cognition in older adults are more frequent. In a study of 1,000 community dwelling adults aged 75 and older, individuals in the lowest quartile for vitamin B12 status scored worse on the Mini Mental State Examination, compared to the top quartile [(140)]. A large study with Chicago residents over the age of 65 showed a statistically significant negative correlation between markers of vitamin B12 status (methylmalonic acid, cystathione, and 2-methylcitrate) with episodic

memory scores and total brain volume [141]. In healthy male participants of the Veteran Affairs Normative Aging Study, both baseline plasma vitamin B12 and dietary recall assessed vitamin B12 intake were associated with increased constructional praxis abilities as assessed by spatial drawing three years after baseline measures [142]. This positive relationship may be due to individuals with low levels of vitamin B12. In normo-cognitive community dwelling individuals aged 75-96 in Sweden, individuals with low vitamin B12 and low folate showed significantly worse scores on free-recall memory compared to those that had normal vitamin B12 and folate status. However, there was no linear relationship between vitamin B12 or folate and episodic memory [143].

Vitamin B12 and AD

It is crucial for the body to clear homocysteine that is produced in the methionine homocysteine cycle, as elevated homocysteine is linked to AD and other diseases including cardiovascular disease, Parkinson's disease, depression, Crohn's disease, inflammatory bowel disease and numerous types of cancers [144].

The literature surrounding the role of vitamin B12 status and supplementation in MCI or AD has been mixed. On the one hand, vitamin B12 supplementation may slow brain atrophy in MCI when Omega-3 fatty acid levels are sufficient [145]. The same study also reported an improvement in executive function in participants who were given vitamin B12 supplements; additionally, in participants with high levels of homocysteine, supplementation led to improved global cognition, episodic memory, and semantic memory [146]. A separate study showed that individuals in the top quintile for B12 intake showed

increased performance in working memory, but no differences in memory or executive function tests [147].

However, many vitamin B12 supplementation trials have not been successful in improving brain structure and function. Vitamin B12 supplementation in aged, cognitively normal adults with diabetes led to less grey matter volume in the left middle temporal pole and the left insula, two areas that are implicated in AD [148]. Similarly, aged adults with mildly elevated plasma homocysteine levels showed less total brain volume after 2 years of daily supplementation with 500 μg of vitamin B12 versus placebo tablet [149]. Folate, vitamin B6 and vitamin B12 supplementation in participants with AD that had normal B12, folic acid, and homocysteine levels led to reduced homocysteine levels but similar cognitive scores to placebo participants [150]. A startling finding of this study was that individuals who were receiving the supplements had significantly higher adverse events in the form of depressive symptoms [150].

Taking into account the mixed literature, it is of interest to determine if serum vitamin B12 levels in non-supplemented individuals may serve as an effective biomarker for AD-related outcomes.

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CHAPTER 3. AUTOTAXIN IS RELATED TO METABOLIC DYSFUNCTION AND PREDICTS ALZHEIMER'S DISEASE OUTCOMES

Modified from a manuscript published in the Journal of Alzheimer's Disease

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Abstract

Background: Obesity and insulin resistance are associated with neuropathology and cognitive decline in Alzheimer's disease (AD). **Objective**: Ecto-nucleotide pyrophosphatase/phosphodiesterase 2, also called autotaxin, is produced by beige adipose

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[*] Shared first authorship

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

tissue, regulates metabolism, and is higher in AD prefrontal cortex (PFC). Autotaxin may be a novel biomarker of dysmetabolism and AD. **Methods**: We studied Alzheimer's Disease Neuroimaging Initiative participants who were cognitively normal (CN; n=86) or had mild cognitive impairment (MCI; n=135) or AD (n=66). Statistical analyses were conducted using SPSS software. Multinomial regression analyses tested if higher autotaxin was associated with higher relative risk for MCI or AD diagnosis, compared to the CN group. Linear mixed model analyses were used to regress autotaxin against MRI, FDG-PET, and cognitive outcomes. Spearman correlations were used to associate autotaxin and CSF biomarkers due to non-normality. FreeSurfer 4.3 derived mean cortical thickness in medial temporal lobe and prefrontal regions of interest. **Results**: Autotaxin levels were significantly higher in MCI and AD. Each point increase in log-based autotaxin corresponded to a 3.5 to 5 times higher likelihood of having MCI and AD, respectively. Higher autotaxin in AD predicted hypometabolism in the medial temporal lobe [R^2=0.343, p<0.001] and PFC [R^2=0.294, p<0.001], and worse performance on executive function and memory factors. Autotaxin was associated with less cortical thickness in PFC areas like orbitofrontal cortex [R^2=0.272, p<0.001], as well as levels of total tau, p-tau$_{181}$, and total tau/Aβ_{1-42}. **Conclusions**: These results are comparable to previous reports using insulin resistance. CSF autotaxin may be a useful dysmetabolism biomarker for examining AD outcomes and risk.

Introduction

Recent studies link obesity and metabolic dysfunction to decreased gray matter volume, less glucose metabolism, and other factors that contribute to Alzheimer's disease (AD)[1-4]. Obesity may give rise to cerebral ischemia, which in turn is associated with brain

tissue degeneration [5]. Additionally, obese individuals are more likely to have higher insulin resistance (IR), defined as the progressive inability for insulin to bind to its receptor [6]. IR is correlated with temporal and frontal amyloid deposition and brain atrophy in late middle-aged participants at risk for AD [2, 7], as well as less glucose metabolism in cognitively normal (CN) elders [8] and AD participants [1]. Resulting oxidative stress may induce cell damage in the brain [9].

Numerous studies have also linked obesity with neuroinflammation and subsequent neurodegeneration, as induced by blood-brain barrier damage or microglial activation in the hippocampi of mice [9, 10]. Rats fed a high fat diet showed increased microglial activation as well as higher levels of the chaperone Hsp72, which are neuroprotective responses to brain injury [11]. Such high fat feeding also upregulates the expression of proinflammatory cytokines in the hypothalamus of rodents [12]. High levels of C-reactive protein, an inflammation marker, have been associated with increased white matter damage in individuals free of dementia [13]. Amylin oligomers and plaques were present in diabetic patients' temporal lobe gray matter and absent in controls [14]. In a subsequent study, rats that overexpressed human amylin in the pancreas had elevated levels of brain pro-inflammatory cytokines, as well as suppressed anti-inflammatory expression [15].

While it is increasingly clear that metabolic dysregulation may play a role in AD, there are currently no established biomarkers in cerebrospinal fluid (CSF) that may reflect central metabolic dysfunction. CSF insulin may not be a reliable biomarker because individuals with type 2 diabetes show reduced insulin uptake in the brain, potentially due to reduced transport across the blood-brain barrier when an individual maintains constant

high blood insulin levels [8]. Ecto-nucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), also known as autotaxin, is an enzyme derived from beige adipose that aids in glucose metabolism regulation and adipose tissue expansion [16] and has been shown to be elevated in the brains of AD subjects versus controls [17]. Autotaxin expression is an outcome of the accumulation of triglycerides in the adipocyte [18]. Autotaxin is mostly expressed in the human brain, placenta, ovary, and small intestine, and stimulates the formation of lysophosphatidic acid (LPA) [19].

In the central nervous system, leptomeningeal cells in the pia mater, as well as astrocytes and oligodendrocytes, are hypothesized to secrete autotaxin [20]. LPA is a lipid-based growth factor and signaling molecule found in all mammalian cells, which promotes the generation of tissue fibrosis *in vivo* and *in vitro*, as well as neurotransmitter release and cell contraction and aggregation via utilization of G protein-coupled receptors [21, 22]. Rat neuronal cell culture work indicates that LPA administration leads to cortical folding and premature differentiation of cells [22]. Normal weight mice who were injected with LPA showed deficits in insulin response to glucose, and pancreatic beta-cells showed impaired insulin release when exposed to LPA [23]. Intriguingly, rats administered LPA showed increased blood-brain barrier permeability, suggesting that the effects of elevated autotaxin levels may be due to central and systemic factors [24]. EDG-2, an LPA receptor gene, is overexpressed in growing preadipocytes and may be an important regulator of adipose tissue development [25]. Obese women display positive correlations between serum autotaxin and fasting glucose [26]. Curiously, body mass index (BMI) and autotaxin showed

no significant correlation in this cohort, indicating that autotaxin may be more of a product of IR, as opposed to the extent of obesity in an individual.

Importantly, some evidence suggests that autotaxin may play a role in AD, whereas the role of other metabolic biomarkers may be less clear. Individuals with AD-like dementia showed significantly higher gene expression of autotaxin in the frontal cortex, as compared to control brains [17]. Additional research is warranted to elucidate the association of high autotaxin levels with established AD biomarkers, cognitive function, and prefrontal cortex (PFC) and medial temporal lobe (MTL) brain volume and glucose metabolism in AD. PFC was a target area because our previous associations with IR are most consistent in that region [7, 8, 27-29], and that obesity across the lifespan is consistently associated with prefrontal atrophy [3]. Additionally, autotaxin in AD has only been examined in frontal cortices [17]. MTL is relevant to AD onset and progression, and we have shown that it is also sensitive to metabolic dysfunction.

In this study, data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) were used to examine CSF autotaxin peptide levels among 287 individuals (75.06 ± 6.89 years of age) who were diagnosed as being CN or having late mild cognitive impairment (MCI) due to AD or early AD. Autotaxin was regressed against cortical thickness (CT) and fluorodeoxyglucose (FDG) values in PFC and MTL, to examine if autotaxin was related to frontal and temporal atrophy and hypometabolism akin to our findings with IR [7, 8, 27, 28]. Relationships with neuropsychological function and traditional CSF biomarkers were also ascertained. The purpose of this study was to determine if CSF-derived autotaxin may be a

biomarker for metabolic dysfunction in the brain and could be associated with AD neurological outcomes.

Materials and Methods

Participants

Data from 287 adults aged 56 to 89 were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of MCI and early AD. For up-to-date information, see http://www.adni-info.org. Written informed consent was obtained from all ADNI participants at their respective ADNI sites. The ADNI protocol was approved by site-specific institutional review boards. Baseline mass spectrometry data for autotaxin was available for 86 CN, 135 MCI, and 66 AD participants. By month 24, MCI participants were classified as either remaining stable (MCI-S, n=80) or progressing to AD (MCI-P, n=55). CN subjects had MMSE scores between 24 and 30, a CDR score of 0, and no dementia. Participants were classified as MCI if they had an MMSE score between 24 and 30, a CDR score of 0.5, complaints of memory loss, and objective memory loss measured on the Wechsler Memory Scale Logical Memory II. Probable AD was defined as MMSE scores between 20 and 26, a CDR score of 0.5 or 1.0, and meeting NINCDS/ADRDA criteria for probable AD. For additional information, see ADNI Procedures Manual (http://adni.loni.usc.edu). Details of the consensus procedure by the ADNI Conversion

Committee are described elsewhere[1]. Other baseline data included demographics, structural MRI for PFC and MTL CT, neural glucose metabolism as determined by FDG uptake, APOE4 genotype, CSF and serum biomarkers, BMI, and neuropsychological performance. While amyloid imaging was also of interest, baseline scans were not analyzed due to small sample size (n=20).

Mass spectrometry and fasting glucose

Data were downloaded from the Biomarkers Consortium CSF Proteomics MRM dataset. As described previously[29], the ADNI Biomarkers Consortium Project investigated the extent to which selected peptides, measured with mass spectrometry (MS), could discriminate among disease states. Briefly, Multiple Reaction Monitoring-MS (MRM-MS) was used for targeted quantitation of 567 peptides representing 221 proteins in a single run (Caprion Proteome Inc., Montreal, QC, Canada). Analyte values of ENPP2 are in arbitrary signal units on a natural log scale. Fasting insulin was assayed using a plasma multiplex immunoassay panel (http://adni.loni.usc.edu/). Fasting glucose was derived from a standard laboratory test. Insulin and glucose were used to calculate the homeostatic model assessment, HOMA-IR [30]. Analyses for this report focused on the autotaxin peptide WWGGQPLWITATK. This peptide performed better in predicting glucose metabolism and cortical thickness than ENPP2 SYPEILTLK in preliminary stepwise regression analyses. To confirm that autotaxin reflected central dysmetabolism, established metabolic and inflammatory biomarkers in CSF were regressed against autotaxin, including insulin-like growth factor binding protein 2 (IGFBP2) peptides, pyruvate kinase isozymes M1/M2, neurosecretory protein VGF, fructose-bisphosphate aldolase A, cholecystokinin and IL-6

receptor [31, 32]. These were the only metabolic or metabolic-related indices available in the CSF for ADNI.

APOE genotype

The ADNI Biomarker Core at the University of Pennsylvania conducted APOE ε4 genotyping. We characterized participants as being "non-APOE4" (i.e., zero APOE ε4 alleles) or "APOE4" (i.e., one to two APOE ε4 alleles).

Amyloid and tau CSF biomarkers

CSF sample collection, processing, and quality control of p-tau$_{181}$, total tau, and Aβ_{1-42} are described in the ADNI1 protocol manual (http://adni.loni.usc.edu/) and Shaw et al. [33].

Clinical and cognitive assessments

Baseline global cognition and assessment scores included the Mini-Mental State Examination (MMSE), clinical dementia rating-sum of boxes (CDR-sob), and AD assessment scale-cognitive subscale 11 (ADAS-cog11). The ADNI1 procedures manual describes diagnostic criteria (http://adni.loni.usc.edu/). The various tests that comprise executive function and memory factor are described elsewhere[34].

MRI

T1-weighted images, collected on 1.5T MR imaging units with a resolution of 1.25 x 1.25 x 1.25 mm [35], were pre-processed using FreeSurfer 4.3 [36]. As described previously [2], this software corrects for motion, deskulls, bias corrects, segments, and parcellates gray and white matter into labeled cortical areas. We chose to examine CT instead of volume, as CT is typically considered a more sensitive index of gray matter pathology in participants

who are at-risk [37] or have AD [38]. To contain type 1 error, we first explored one broad region of interest (ROI) in bilateral prefrontal cortex and one ROI in bilateral medial temporal lobe, followed by finer grained associations in sub-ROI if an initial omnibus analysis in a given broad ROI was significant. In the PFC, bilateral sub-ROIs included: 1) pars orbitalis; 2) pars triangularis; 3) caudal middle frontal; 4) rostral middle frontal; 5) superior frontal; 6) frontal pole; 7) lateral orbitofrontal; and 8) medial orbitofrontal. These areas are very similar to ROI chosen in previous reports on metabolic dysfunction with regional amyloid [2] and FDG [1]. In the MTL, bilateral sub-ROI included hippocampal volume (where CT is not calculated by FreeSurfer) and CT in: 1) medial temporal gyrus; 2) parahippocampal gyrus; and 3) entorhinal cortex. Bilateral pre-central gyrus was chosen as a control ROI, an area that shows little association with dysmetabolism and brain atrophy in middle-aged participants [7] or glucose metabolism in AD[1].

FDG-PET

Acquisition is described elsewhere [27, 39]. Images were resliced to a 1.5 mm^3 voxel resolution in a 160 x 160 x 96 spatial matrix, intensity normalized to pons to derive the standardized uptake volume ratio (SUVR), smoothed using an 8mm Gaussian kernel, and normalized to Montreal Neurological Institute space. Due to the poorer native resolution of the FDG-PET images and smoothing to reduce noise, only the two broad ROI in PFC and medial temporal lobe were used. Similarly broad ROI have been used to examine relationships between dysmetabolism and FDG-PET [27].

Statistical analyses

All statistical analyses were conducted using SPSS 23.0 software (IBM Corp., Armonk, NY). All analysis models except for cognition included the following covariates: age at baseline, sex, BMI, APOE ε4 genotype, and either baseline diagnosis or MCI conversion. The random effect of Subject was also covaried. For executive function, memory and global assessments, education was added as a covariate but not baseline diagnosis or MCI conversion, because cognitive assessments directly inform how participants are clinically classified. Linear mixed models, followed by least significant differences (LSD) post-hoc tests, were used to test if autotaxin levels differed by baseline diagnosis (CN, MCI, AD) or MCI conversion (MCI-S or MCI-P). Multinomial regression analyses tested if higher autotaxin was associated with higher relative risk ratios for an MCI or AD diagnosis, with CN as the reference group. Logistic regression was similarly used to assess increased risk of being MCI-P relative to MCI-S based on autotaxin.

Mixed model analyses were also performed to regress autotaxin against metabolic, MRI, FDG-PET, and cognitive outcomes. The main effect of autotaxin and its interaction with baseline diagnosis or MCI conversion were tested in a given model. Previous work has suggested that systemic IR is associated with neural outcomes like less regional FDG-PET in AD, but show different relationships in MCI-S or MCI-P depending on the brain region [27]. For the MRI analyses, to robustly contain type 1 error, an initial multivariate repeated measures omnibus was initially conducted for a broad PFC or medial temporal ROI, as described elsewhere [40], where each ROI was composed of several sub-ROI selected a priori based on our prior work [1, 2]. Briefly, this technique determines if there is an overall

significant association with all sub-ROIs, allowing subsequent analyses to investigate each sub-ROI to determine where omnibus signal was derived without Bonferroni or similar corrections [41]. Even so, as a further type 1 error check described by Willette et al. [27], Holm-Bonferroni correction [42] was used to adjust the family-wise error rate to 0.05. Specifically, for a significant follow-up interaction for a given sub-ROI, autotaxin levels were regressed against CT for CN, MCI, and AD separately, or 3 null hypotheses per ROI. A p value of 0.017 was needed among one of the groups to achieve significance, followed by 0.025 and 0.050.

Lastly, as described by the ADNI Biomarker Core [29], CSF and plasma analyte values were log-transformed to achieve normality. With the exception of AD biomarkers, all variables had homoscedastic variance and a normal distribution. Due to violations in regression diagnostics for p-tau$_{181}$, total tau, and A$\beta_{1\text{-}42}$ (see below), the non-parametric Spearman's statistic was used to correlate autotaxin and CSF biomarker values.

Results

Demographics and data summary

Summary information is listed in Table 3.1. All variables were normally distributed except for A$\beta_{1\text{-}42}$ ($D=0.130$, $p<0.001$), Total tau ($D=0.121$, $p<0.001$), and p-tau ($D=0.104$, $p<0.001$), as fit against normal Q-Q plots was non-linear. Data transforms did not resolve non-normality. Non-parametric correlations were therefore conducted between autotaxin and CSF amyloid and tau biomarkers.

Baseline diagnosis: differences in autotaxin levels

Linear mixed models showed a main effect of baseline clinical diagnosis on autotaxin [$F(2,286)=3.100$, $p=0.047$]. There was a significant increase in log-scaled autotaxin from CN to either MCI [Mean Difference ± SE=0.078±0.036, $p=0.029$] or AD [Mean Difference ± SE=0.094±0.043, $p=0.029$], but not between MCI and AD [Mean Difference ± SE=0.016±0.038, $p=0.665$].

As a follow-up analysis, multinomial logistic regression was then used to examine if CSF autotaxin expression predicted an increased likelihood of being MCI or AD. The reference group was CN. The likelihood ratio statistic [$\chi^2=5.990$, $p=0.050$] indicated that higher autotaxin levels predicted a higher Odds Ratio for being MCI [β±SE=1.248±0.605, OR=3.485, 95% CI=1.065 to 11.397, Wald=4.264, $p=0.039$] or AD [β±SE=1.597±0.747, OR=4.940, 95% CI=1.134 to 21.351, Wald=4.574, $p=0.032$]. These results suggest that autotaxin levels were higher in memory impaired versus CN participants, and that per point increase in log-based autotaxin values corresponded to a roughly 3.5 to 5 times increase in the odds of having some degree of clinically relevant memory impairment. For MCI conversion by 24 months, there was no significant difference between MCI-S and MCI-P. Baseline autotaxin levels also did not predict likelihood of MCI conversion [$\chi^2=0.177$, $p=0.674$].

Associations of autotaxin with metabolic and inflammatory indices

Next, to validate autotaxin as an energy metabolism biomarker, CSF autotaxin levels were regressed against total body mass, peripheral and CSF metabolic and inflammatory biomarkers (Table 3.2), as well as used to predict likelihood of having pre-diabetes or type 2

diabetes. We found that higher autotaxin was related to higher fasting glucose, several established biomarkers of metabolism in the CSF, CSF interleukin-6, and that higher levels per point increase reflected a 300% greater likelihood of having pre-diabetes or type 2 diabetes.

ROI analysis: PFC and MTL CT

For bilateral PFC CT, a multivariate omnibus was conducted in one broad PFC ROI incorporating several sub-ROI from FreeSurfer areas, to minimize type 1 error [41]. The main effect of autotaxin and its interaction with baseline diagnosis were investigated. The interaction tested if autotaxin differentially predicted less CT among CN, MCI, or AD participants. There was a marginal, within-subject Regions x Baseline Diagnosis x Autotaxin interaction after Huynh-Feldt correction [$F(14,1200)=1.784$, $p=0.066$], which became significant after removing one outlier with a high autotaxin value (18.31) [$F(14,1197)=2.031$, $p=0.032$]. As indicated in Table 3.3, even after applying Holm-Bonferroni correction, higher autotaxin was associated with thinner CT in most PFC sub-ROI for AD participants, such as medial orbitofrontal cortex (Fig. 3.1A) and only in Pars Orbitalis for MCI, while the CN group showed negative and positive associations. By contrast, the omnibus for the one broad medial temporal ROI was non-significant, where further analyses were not pursued to guard against type 1 error. In the pre-central gyrus control region, there was no significant main effect of autotaxin or interaction with baseline diagnosis.

ROI analysis: PFC and MTL glucose metabolism

For FDG-PET, an Autotaxin * Baseline Diagnosis interaction [$F(2,146)=3.106$, $p=0.048$] showed that higher autotaxin predicted less glucose uptake in the broad PFC ROI

for AD subjects [β±SE=-0.127±0.027, t(1,35)=-4.708, p<0.001], marginally for CN [β±SE=-0.074±0.041, t(1,35)=-1.822, p=0.077] and not for MCI [β±SE=0.043±0.029, t(1,70)=1.497, p=0.139]. Removal of the same autotaxin outlier from the PFC CT analysis did not affect the association in the AD group [β±SE=-0.116±0.032, T(1,34)=-3.610, p=0.001] (Fig. 3.1B). In addition, the Autotaxin * MCI Conversion interactions were non-significant for PFC.

Similar relationships were seen in the broad temporal ROI. A Baseline Diagnosis * Autotaxin interaction [F(2,146)=14.175 p<0.001] revealed that higher autotaxin was associated with less MTL glucose metabolism in AD [β±SE=-0.586±0.026, t(1,37)=-4.334 p<0.001] and CN participants [β±SE=-0.429±0.049, t(1,35)=-2.773 p=0.009], but not in MCI [β±SE=0.189±0.027, t(1,72)=1.619 p=0.110].

FDG uptake in the pre-central gyrus control region did not show a main effect of autotaxin or interaction with baseline diagnosis or MCI conversion.

Cognition: global and executive function factor scores

As shown in Table 3.4, separate linear mixed models indicated that per point increase in autotaxin, scores were lower for the executive function and memory factors, but not for global cognition and function scores.

CSF biomarkers: $A\beta_{1-42}$, total tau, and p-tau

Across all participants, the Spearman's statistic showed that higher autotaxin was associated with higher levels of total tau, p-tau_{181}, and total tau/$A\beta_{1-42}$ (Table 3.5).

Discussion

In this study, we hypothesized that CSF-derived autotaxin may be a biomarker for brain metabolic dysfunction relevant to AD. Strikingly, higher autotaxin predicted less

bilateral PFC and MTL glucose uptake in individuals with AD, with similar effects seen in bilateral PFC CT. Results also showed that participants who had MCI or AD had significant increased log-scaled autotaxin. IR shows a similar relationship with PFC and MTL FDG-PET in late middle-aged participants [28], and among aged CN adults with type 2 diabetes [8] or AD [27], where the ADNI AD group for the previous report and this cohort show similar results.

Higher autotaxin correlated with established metabolic biomarkers and higher risk for pre-diabetes and type 2 diabetes. CSF ENPP2 was moderately associated with peripheral fasting glucose, but not BMI, where others have noted similar findings [26]. Furthermore, per point increase in autotaxin, executive function and memory factor scores were lower. Similarly, a decreased insulin sensitivity index in rhesus monkeys [43] was associated with less PFC gray matter and mediated worse motor-planning executive function performance. Higher HOMA-IR in aged humans with AD was also related to less MTL and PFC glucose metabolism [27], respectively mediating worse memory and executive function factor scores (unpublished data). Several studies have found similar associations between metabolic dysfunction, PFC outcomes, and executive function. Diabetes was significantly associated with worse cognitive performance and attention deficit in young adults without dementia [44]. In a cohort of bariatric surgery patients, as HOMA-IR levels decreased following the surgery, cognition scores improved [45]. Taken together, autotaxin shows a pattern of relationships with established metabolism biomarkers, MTL and PFC-specific outcomes, and related cognitive dysfunction that may reflect central dysmetabolism and is similar to our previous findings with IR.

Autotaxin was also positively correlated with levels of total tau, p-tau$_{181}$, total tau/Aβ_{1-42}, as well as p-tau$_{181}$/Aβ_{1-42} to a marginal degree. This pattern suggests that CSF autotaxin predicts AD neuropathology in a manner similar to dysmetabolism. Chronic application of insulin *in vitro* in human cortical stem cells appeared to induce insulin resistance and inhibit tau dephosphorylation [46]. Higher IR among cognitively normal, APOE4 late middle-aged participants also predicted higher total tau and phosphorylated tau [47], but not CSF amyloid. Amyloidosis is scant in this late middle-aged cohort, but does show modest regional associations with higher IR [2]. ADNI did not have enough baseline amyloid scans (n=20) to warrant analysis with autotaxin.

This brings to question the underlying mechanism behind the correlation of autotaxin and AD-related outcomes. Individuals with high autotaxin may be more likely to be obese, causing an increase in IR, ischemia, and oxidative stress. Results of our study showed a weak association between systemic glucose and CSF autotaxin. As an alternative or interrelated mechanism, autotaxin stimulates the release of LPA, which may be detrimental [21, 22]. LPA has been shown to prompt neurite withdrawal and tau phosphorylation in neuroblastoma cells [48]. Addition of LPA to neuroblastoma cells led to increased immunoreactivity with antibodies that react with tau phosphorylation [17].

There are several study limitations to address. Regression diagnostic issues were encountered for total tau, p-tau$_{181}$, and Aβ_{1-42}, requiring non-parametric analyses. Autotaxin levels did not differ in MCI conversion or contribute to MCI conversion risk, nor were there significant interactions with MCI conversion, which may be due to small sample size. Instead, baseline values were widely dispersed in the MCI converter group. This mirrors IR

MCI interactions [1]. No associations were found with Aβ levels, but there were significant associations with tau and p-tau$_{181}$, as well as tau to amyloid ratios. Given that the mass spectrometry panel was only available at baseline, we were unable to longitudinally assess autotaxin levels and various outcomes. While longitudinal predictions would be useful, it was beyond the scope of this report, which was to systematically assess if autotaxin may be a relevant AD biomarker. While autotaxin was associated with fasting glucose, it was surprising that it was not associated with systemic insulin or HOMA-IR. This may be because insulin was derived from a plasma multiplex, and the values are lower than ELISA. Nonetheless, autotaxin was associated with fasting glucose, risk for pre-diabetes and type 2 diabetes, and was associated with many established metabolic factors in CSF. It also shows a pattern of results comparable to HOMA-IR and either brain atrophy or FDG-PET[7, 27, 28]. The non-significance of MTL ROI CT was unexpected; however, it also provides direction for future research. Glucagon-like peptide-1 (GLP-1) may be a potential element in this phenomenon. GLP-1 stimulates the release of insulin as well as downregulates the release of glucagon and breakdown of amyloid-β protein precursor [49]. GLP-1 receptor production in the aged mouse medial PFC was decreased compared to young mice, but no similar relationship was seen in the hippocampal regions [49].

In summary, this study demonstrates that CSF-derived autotaxin in elders correlated with greater odds for having MCI or AD, hyperglycemia, AD neuropathology related to total tau, p-tau$_{181}$, and the total tau/Aβ_{1-42} ratio, less gray matter CT in PFC, hypometabolism in PFC and MTL, and worse executive function and memory scores. These results support the theory that autotaxin may be an indicator of central dysmetabolism for a variety of AD

outcomes. Further replication is necessary to determine if autotaxin predicts AD and tracks certain aspects of the disease. Future clinical and animal model work should consider examining this biomarker and its applications as a potential target for pharmacologic therapies in the setting of glucose dysregulation and AD. Such work should also examine how autotaxin predicts changes in these outcomes over time.

Authors' contributions

K.E.M. conducted data analysis and manuscript preparation. A.A.W. conducted data analysis and manuscript preparation.

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California. The data used in the preparation of this article were obtained from the ADNI database (http://adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report.

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Tables and Figures

Table 3.1. Demographics and sample characteristics

	CN	MCI	AD	MCI-S	MCI-P
Age	75.70 ± 5.54	74.69 ± 7.35	74.98 ± 7.57	74.74 ± 6.96	74.63 ± 7.94
Female	42	44	29	25	19
Male	44	91	37	55	36
Education	15.64 ± 2.97	16.00 ± 2.96	15.11 ± 2.96	16.33 ± 2.91	15.51 ± 3.00
APoE ε4-	65	64	19	41	23
APoE ε4+	21	71	47	39	52
CDR-sob	0.02 ± 0.11	1.56 ± 0.88	4.34 ± 1.56	1.41 ± 0.79	1.77 ± 0.97
MMSE	29.05 ± 1.02	26.91 ± 1.74	23.52 ± 1.85	27.35 ± 1.62	26.27 ± 1.72
ADAS-cog11	6.05 ± 2.90	11.72 ± 4.33	18.88 ± 6.71	10.70 ± 4.05	13.19 ± 4.32
Memory factor	0.98 ±0.50	-0.15±0.57	-0.91±0.55	0.04 ± 0.57	-0.43 ± 0.45

Numbers represent frequency or unadjusted mean ± SD. MCI-S, Stable MCI at 12 months.

MCI-P, Progression to AD from MCI at 12 months.

Table 3.2. The association of autotaxin with CSF metabolic and inflammatory biomarkers.

	β ± SE	F value	p value
Fructose-bisphosphate aldolase A	0.415±0.097	18.551	<0.001
Cholecystokinin	0.378±0.123	9.369	0.002
Pyruvate Kinase Isozymes M1/M2	0.576±0.108	28.364	<0.001
Neurosecretory protein VGF	0.574±0.162	12.614	<0.001
IGFBP2	0.595±0.056	114.899	<0.001
IL-6 receptor			
CN	0.102±0.011	93.830	<0.001
MCI	0.067±0.010	45.705	<0.001
AD	0.097±0.015	41.301	<0.001

Model estimate beta values and SE of autotaxin for CSF metabolites and inflammation biomarkers.

Table 3.3. The interaction of clinical diagnosis and autotaxin on prefrontal cortex thickness.

	CN		MCI		AD	
Cortical Thickness	**T value**	**β ± SE**	**T value**	**β ± SE**	**T value**	**β ± SE**
Pars Orbitalis	-1.519	-0.073 ± 0.048	-2.188	-0.073 ± 0.034*	-4.253	-0.230 ± 0.054***
Pars Triangularis	-4.300	-0.062 ± 0.014***	-1.914	-0.041 ± 0.021	-4.178	-0.102 ± 0.024***
Rostral Middle Frontal	-0.073	-0.001 ± 0.013	-1.592	-0.028 ± 0.018	-4.180	-0.090 ± 0.022***
Lateral Orbitofrontal	3.214	0.046 ± 0.014**	-0.260	-0.005 ± 0.020	-5.306	-0.122 ± 0.023***
Medial Orbitofrontal	1.178	0.020 ± 0.017	-0.664	-0.014 ± 0.021	-4.922	-0.127 ± 0.026***
Caudal Middle Frontal	-1.208	-0.015 ± 0.013	1.146	0.021 ± 0.019	0.667	0.014 ± 0.021
Superior Frontal	-3.596	-0.062 ± 0.017**	0.178	0.004 ± 0.025	-1.705	-0.050 ± 0.029
Frontal Pole	1.153	0.023 ± 0.020	1.378	0.034 ± 0.025	-10.925	-0.344 ± 0.031***

*p<0.05, **p<0.01, ***p<0.001

Model estimate beta values and SE of autotaxin for prefrontal CT ROI. T values are for each clinical group. For each ROI, Holm-Bonferroni correction required successive p values of 0.017, 0.025, and 0.050 for a given diagnostic group fit-line to be considered significant.

Table 3.4. The association of autotaxin with cognitive indices.

Cognitive Parameter	F value	p value	β ± SE
ADAS-cog11	1.251	0.264	1.802 ± 1.611
CDR-sob	0.831	0.363	0.405 ± 0.444
Executive Factor	**4.215**	**0.037**	**-0.464 ± 0.222**
Memory Factor	**4.222**	**0.041**	**-0.333 ± 0.056**
MMSE	0.054	0.816	0.230 ± 0.606

Model estimate beta values and SE of autotaxin for global indices and the executive function and memory factors. Bolded text indicates results with a significant p value.

Table 3.5. The association of autotaxin and AD CSF markers.

CSF Biomarker	p value	Spearman's R value
$A\beta_{1\text{-}42}$	0.738	-0.020
p-tau$_{181}$	**0.028**	**0.130**
p-tau$_{181}$/$A\beta_{1\text{-}42}$	0.070	0.108
Total Tau	**0.002**	**0.185**
Total Tau/$A\beta_{1\text{-}42}$	**0.018**	**0.141**

Due to regression diagnostics, parametric models were not conducted between autotaxin and CSF biomarkers. Bolded text indicates results with a significant p value.

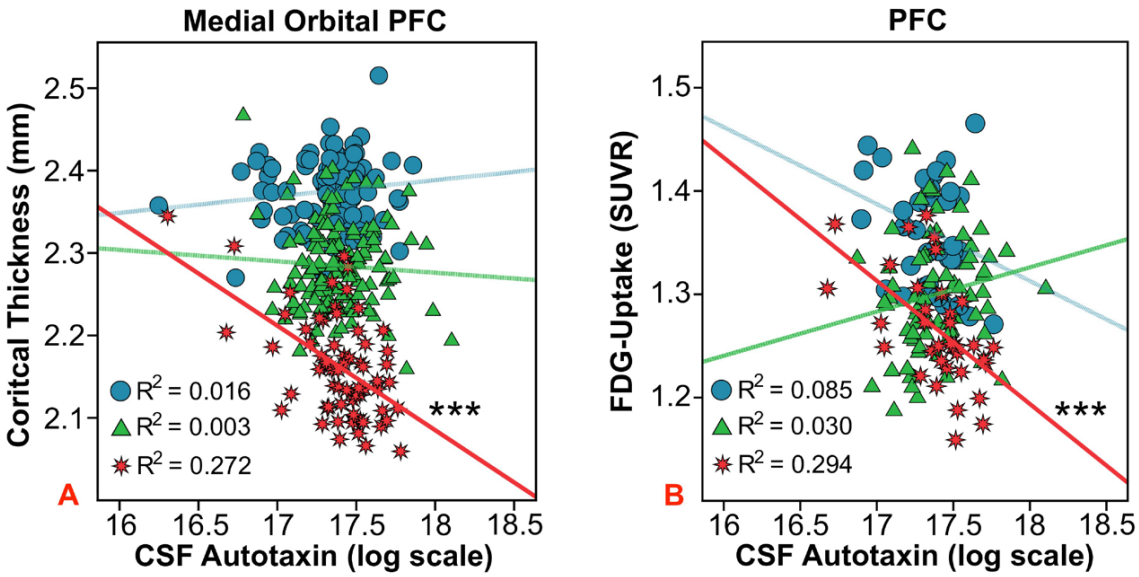

Figure 3.1. Autotaxin and PFC region of interest analyses. The association between CSF autotaxin and baseline bilateral medial orbital PFC cortical thickness (A) or bilateral PFC FDG-PET glucose uptake (B), an index of glucose metabolism, among baseline diagnosis groups. The "blue circle", "green triangle", and "red star" symbols correspond to CN, MCI, and AD participants respectively. The R^2 value refers to the proportion of variance in CT or FDG-PET uptake explained by autotaxin for a given group. ***$p < 0.001$.

CHAPTER 4. PERIPHERAL VERSUS CENTRAL INDEX OF METABOLIC DYSFUNCTION AND ASSOCIATIONS WITH CLINICAL AND PATHOLOGICAL OUTCOMES IN ALZHEIMER'S DISEASE

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Abstract

Background/Objective: Insulin-like growth factor binding protein 2 (IGFBP-2) regulates blood glucose levels, facilitates hippocampal synaptic plasticity and may have a predictive value for Alzheimer's disease (AD) diagnosis. **Methods:** IGFBP-2 levels were studied in plasma in 566 subjects and in cerebrospinal fluid (CSF) in 245 subjects across the AD spectrum from the Alzheimer's Disease Neuroimaging Initiative (ADNI). Variants in the *IGFBP-2* gene were examined. Linear mixed modeling in SPSS tested main effects of IGFBP-2 and interactions with APOE4 on neurocognitive indices and biomarkers. Voxel-wise regression was used to gauge IGFBP-2 and regional gray matter and glucose metabolism associations. **Results:** Each point increase in IGFBP-2 corresponded to a 3 times greater likelihood of having mild cognitive impairment (MCI) or AD. IGFBP-2 showed beneficial associations with respect to cognitive scores in individuals with two APOE4 alleles. Higher IGFBP-2 predicted higher insulin resistance, but not CSF amyloid or tau. Voxel-wise analyses showed that plasma IGFBP-2 predicted lower grey matter volume and FDG metabolism in a large area spanning the frontal, temporal, and occipital lobes. CSF IGFBP-2 levels showed similar voxel-wise analysis results but were uniquely associated with CSF amyloid and tau. Analysis of single nucleotide polymorphisms (SNPs) in IGFBP-2 showed that subjects carrying risk alleles versus common alleles had increased risk of AD and lower memory scores. Voxel-wise analyses of these SNPs also implicated the hippocampus and prefrontal cortex. **Conclusions:** IGFBP-2 is associated with AD risk and outcomes; plasma IGFBP-2 provides stronger predictive power for brain outcomes, while CSF IGFBP-2 provides improved predictive accuracy for AD CSF biomarkers.

Introduction

Insulin-like growth factor binding proteins (IGFBP) serve to bind insulin-like growth factors (IGF) to regulate their concentrations and consequently metabolic activity [1]. IGFBPs are central for the transport of IGF to its receptor site, and have the ability to enhance binding to the receptor site or inhibit the access of IGF to its receptors by creating strong bonds, lowering binding potential [2]. IGFBP is centrally important in regulating blood glucose levels, although in excess can be a marker of disease. IGFBP-2, in particular, binds to both IGF-1 and IGF-2, and can inhibit IGF functions such as DNA synthesis, cell proliferation, and cell death, as well as glucose and amino acid uptake in cells [3, 4]. For example, transgenic mice that overexpressed IGFBP-2 showed significantly higher blood glucose levels than controls at 30, 60 and 90 minutes during an oral glucose tolerance test, as well as lower levels of GLUT4--the insulin-dependent glucose transporter--at cell surfaces compared to controls [5]. Abnormally high IGFBP-2 levels are often an indicator of severe catabolic events, such as gastric cancer, anorexia nervosa and renal failure [6-8]. In a study of 625 men and women aged 70 and older, plasma IGFBP-2 significantly predicted mortality from all causes after adjusting for markers of body composition, as well as fasting glucose and insulin [9].

Alzheimer's disease (AD) has been associated with defects in the insulin signaling pathway. Higher levels of insulin resistance (IR) were correlated with increased regional amyloid deposition and atrophy in frontal and temporal areas of the brain in late middle-aged adults [10, 11]. In addition, higher IR was associated with decreased cerebral glucose metabolic rate and worse memory scores in cognitively normal adults with pre-diabetes or

type 2 diabetes [12]. Animal models provide evidence that IGFBP-2 may play a role in AD. Over-expression of IGFBP-2 in mice led to decreased weights of the hippocampus, cerebellum, olfactory bulb and prefrontal cortex at 12 weeks, compared to controls [13]. An important study in humans utilized ex vivo tissue from individuals across the AD spectrum, and showed that IGF resistance was related to beta amyloid (Aβ) plaques, other markers of AD, and worse ante-mortem cognition [14]. Some associations may be compartment-specific, as CSF but not plasma IGFBP-2 levels were higher in 92 AD patients versus 72 healthy controls [15], where CSF IGFBP-2 levels were positively correlated with total tau and phosphorylated-181-tau (p-tau). Also, APOE4 alleles have been shown to play a role in insulin metabolism and cognitive function [16]. Cognitively normal (CN) and AD participants who were APOE4 homozygous showed improvements in memory with lower doses of intranasal insulin as compared to individuals who were not APOE4 homozygous [16]. Additionally, a study using euglycemic hyperinsulinemic clamps in individuals with impaired glucose tolerance and healthy controls showed that IGFBP-2 was the only insulin signaling molecule assayed to independently predict bioactive levels of IGF-1 in both groups; this study showed a negative correlation between IGFBP-2 and IGF-1 [17].

As biomarkers become increasingly more important in tracking AD diagnosis and progression, and metabolic markers have gained more interest, additional research is needed to explore the role of IGFBP-2 in AD. We hypothesize that IGFBP-2 levels would be positively associated with more brain atrophy, less neuronal glucose uptake, and impaired cognitive function. In this study, we utilized data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) to examine plasma levels of IGFBP-2 among 566 individuals

that were cognitively normal (CN), or had mild cognitive impairment (MCI) or AD. This is the first study, to our knowledge, to systematically study the relationship between peripheral IGFBP-2 levels and related single nucleotide polymorphisms (SNPs) with neural, cognitive, and biomarker outcomes relevant to AD, including peripheral inflammatory markers like Interleukin-6 (IL-6) receptor and C-peptide. On an exploratory basis in a subset of 245 subjects, we also examined how CSF mass spectrometry IGFBP-2 peptide was related to these outcomes as compared to plasma IGFBP-2.

Materials and Methods

Participants

Data from middle-aged to aged adults were obtained from the ADNI database (http://adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of MCI and early AD. For up-to-date information, see http://www.adni-info.org. Written informed consent was obtained from all ADNI participants at their respective ADNI sites. The ADNI protocol was approved by site-specific institutional review boards. All analyses used in this report only included baseline data. Baseline plasma data for IGFBP-2 was available for 566 participants: 58 CN, 396 MCI, and 112 AD. Baseline CSF data for IGFBP-2 was available for 245 subjects: 45 CN, 134 MCI, and 66 AD. Baseline genomic data was available for 756 participants: 229 CN, 354 MCI, and 173 AD.

Participants with MCI had the following diagnostic criteria: 1) memory complaint identified by the participant or their study partner; 2) abnormal memory as assessed by the Logical Memory II subscale from the Wechsler Memory Scale- Revised, with varying criteria based on years of education; 3) Mini-Mental State Exam (MMSE) score between 24 and 30; 4) Clinical dementia rating of 0.5; 5) Deficits not severe enough for the participant to be diagnosed with Alzheimer's disease by the site physician at screening. Participants with AD met similar criteria, however, were required to have an MMSE score between 20 and 26, a clinical dementia rating of 0.5 or 1.0, and NINCDS/ADRDA criteria for probable AD.

Mass Spectrometry and Fasting Glucose

Data were downloaded from the Biomarkers Consortium CSF Proteomics MRM dataset and the Biomarkers Consortium Plasma Proteomics Project RBM multiplex. As described previously [18], the ADNI Biomarkers Consortium Project investigated the extent to which selected peptides, measured with mass spectrometry, could discriminate among disease states. Briefly, Multiple Reaction Monitoring-MS (MRMMS) was used for targeted quantitation of 567 peptides representing 221 proteins in a single run (Caprion Proteome Inc., Montreal, QC, Canada). Fasting insulin was assayed using a plasma multiplex immunoassay panel (http://adni.loni.usc.edu/), which we note produce consistently lower insulin values than a standard ELISA kit. Fasting glucose was derived from a standard lab test. Insulin and glucose were used to calculate the homeostatic model assessment, HOMA-IR. Analyses for this report focused on IGFBP-2 levels, which were assayed in the plasma multiplex panel and CSF proteomics panel, for which the peptide LIQGAPTIR was chosen,

which performed better in most analyses (data not shown). For proinflammatory markers, C-peptide and IL-6 receptor levels were derived from the plasma multiplex.

APOE Genotype

The ADNI Biomarker Core at the University of Pennsylvania conducted APOE ε4 genotyping. We characterized participants as having zero APOE4 alleles, one APOE4 allele, or two APOE ε4 alleles.

Amyloid and Tau CSF Biomarkers

CSF sample collection, processing, and quality control of p-tau, total tau, and $A\beta_{1-42}$ are described in the ADNI1 protocol manual (http://adni.loni.usc.edu/) and Shaw et al [19].

Neuropsychological Assessment

ADNI utilizes an extensive battery of assessments to examine cognitive functioning with particular emphasis on domains relevant to AD. A full description is available at http://www.adni-info.org/Scientists/CognitiveTesting.aspx. All subjects underwent clinical and neuropsychological assessment at the time of scan acquisition. Neuropsychological assessments included: The Clinical Dementia Rating sum of boxes (CDR-sob), Mini-Mental Status Exam (MMSE), Auditory Verbal Learning Test (RAVLT), and AD Assessment Schedule - Cognition (ADAS-Cog). A composite memory score encompassing the RAVLT, ADAS-COG, MMSE, and Logical Memory assessments was also utilized [20]. Additionally, a composite executive function score comprising Category Fluency—animals, Category Fluency—vegetables, Trails A and B, Digit span backwards, WAIS-R Digit Symbol Substitution, Number Cancellation and 5 Clock Drawing items was used[21]. These composite scores were used in formal analyses to represent global memory and executive function among subjects.

Magnetic Resonance Imaging (MRI) Acquisition and Pre-Processing

T1-weighted MRI scans were acquired within 10-14 days of the screening visit following a back-to-back 3D magnetization prepared rapid gradient echo (MP-RAGE) scanning protocol described elsewhere [22]. Images were pre-processed using techniques previously described [11]. Briefly, the SPM12 "New Segmentation" tool was used to extract modulated gray matter (GM) volume maps. Maps were smoothed with an 8mm Gaussian kernel and then used for voxel-wise analyses.

FDG-PET

FDG-PET acquisition and pre-processing details have been described previously [22]. Briefly, 185 MBq of [18-153-F]-FDG was injected intravenously. After 30 minutes, six 5-minute frames were acquired. Frames of each baseline image series were co-registered to the first frame and combined into dynamic image sets. Each set was averaged, reoriented to a standard 160 x 160 x 96 voxel spatial matrix of resliced 1.5 mm^3 voxels, normalized for intensity, and smoothed with an 8 mm FWHM kernel. In order to derive the standardized uptake value ratio (SUVR), pixel intensity was normalized according to the pons since it demonstrates preserved glucose metabolism in AD [23]. Normalization to the pons removed inter-individual tracer metabolism variability. The Montreal Neurological Institute (MNI) template space was used to spatially normalize images using SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). A subset of subjects underwent FDG-PET scans and analyses included in this report.

Statistical Analysis

All analyses were conducted using SPSS 24 (IBM Corp., Armonk, NY) or SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). Linear mixed effects models tested the main effects of plasma or CSF IGFBP-2 on cognitive scores, biomarkers, Baseline Diagnosis, and their outcomes of interest in SPSS 24. Main effects and interactions with APOE or Baseline Diagnosis were tested in a single model. Covariates included age at baseline, sex, and baseline diagnosis in all models. An additional covariate, years of education, was included when analyzing memory and cognitive performance. Outcomes included neuropsychological performance, modulated GM maps and FDG maps, and CSF biomarkers including $A\beta_{1\text{-}42}$, t-tau and p-tau. Binomial logistic regression was also used to assess the odds ratio of a given participant being diagnosed as MCI or AD versus the CN reference group. Linear regression was used in PLINK to assess genetic associations.

To correct for type 1 error in non voxel-wise analyses, as described previously [24, 25], Holm-Bonferroni correction was used for each set of analyses. This closed test procedure maintains a family-wise P value = 0.05 by requiring unadjusted P values of 0.05 divided by x, x being the number of null hypotheses tested. For 4 cognitive tests, for example, P values of .0125, .025, .0375, and .050 are successively needed among any test to proceed with testing in the closed set. For sets that were not robust under Holm-Bonferroni correction, a less strict form of correction was used. Specifically, omnibus testing using MANCOVA incorporating all dependent variables of the set was conducted, where a significant main effect or Baseline Diagnosis interaction allowed further testing of all outcomes as follow-up tests. A family-wise error rate of .05 is maintained using this approach [26].

For voxel-wise analysis, 2nd-level mixed models tested main effects of IGFBP-2 on regional GM volume and FDG, controlling for age, sex, education, and Baseline Diagnosis. The thresholds were set at $P < .005$ (uncorrected) and $P < .05$ (corrected) for voxels and clusters respectively. Results were considered significant at the cluster level. As described previously [24], in order to reduce type 1 error, we utilized a GM threshold of 0.2 to ensure that voxels with <20% likelihood of being GM were not analyzed. For GM, Monte Carlo simulations in ClusterSim (http://afni.nimh.nih.gov/afni/doc/manual/3dClustSim) were used to estimate that 462 contiguous voxels were needed for such a cluster to occur at $P < 0.05$. For FDG voxel-wise analyses, Monte Carlo simulations in ClusterSim were used to estimate that 224 contiguous voxels were needed for such a cluster to occur at $P < 0.05$.

Genomic Data Processing and Quality Control

Genomic data underwent stringent quality control (QC) by assessing concordance with Hardy-Weinberg equilibrium (HWE) scrutinized data for Mendelian inheritance errors. Single Nucleotide Polymorphisms (SNPs) were filtered based on HWE P-value > 0.00001 and MAF >0.05% and a call rate of 95%. Samples with greater than 5% missingness were removed. Sample genotypes were imputed using 1000Genomes data with Shapeit/Impute2 software following the protocol outlined here [27]. SNPS with call rates <95 % or $R^2 \leq 0.3$ were removed leaving 2,976,223 imputed and genotyped SNPs after quality control. All analyses were conducted using PLINK v1.9 (http:// www.cog-genomics.org/plink2).

Results

Data Summary

Clinical, demographic, and CSF data for subjects with plasma IGFBP-2 are presented in **Table 4.1**. There were no differences based on years of education or age at baseline between CN, MCI or AD subjects. As expected for this ADNI sub-population, there was a significant difference in the percentage of APOE4 carriers and in cognitive function. Subsequently, analyses were conducted with plasma IGFBP-2. Demographic data for subjects with CSF IGFBP-2 are presented in **Supplemental Table 4.1**. See **Supplemental Text 4.1** for analyses that used CSF IGFBP-2 in a subset of ADNI subjects.

Clinical Characteristics and Alzheimer's Disease Risk

Logistic regression was used to examine if plasma IGFBP-2 expression predicted an increased likelihood of being MCI or AD. The reference group was CN. The likelihood ratio statistic [X^2=74.450, p<.001] indicated that higher IGFBP-2 levels predicted a higher Odds Ratio for being MCI or AD [Wald=9.938, β=3.003, p=0.002]. These results suggest that a per point increase in IGFBP-2 corresponded to a roughly 3 times likelihood of having some degree of clinically relevant memory impairment. No significant associations were shown with IGFBP-2 and MCI conversion. No significant interaction was shown between IGFBP-2 and APOE with regards to predicting an odds ratio for being MCI or AD.

Global Cognition, Memory, Visual Spatial, and Executive Function

Linear mixed models showed a non-significant main effect for IGFBP-2 on global indices including CDR-sob, ADAS-cog and MMSE. Among neuropsychological indices depicted in **Figure 4.1**, the number of APOE4 alleles modified how plasma IGFBP2 was

related to global cognition. Specifically, for CDR-sob, a IGFBP-2 * APOE interaction was significant ($\beta \pm SE$= -2.825$\pm$0.983, p=.034). Similarly for ADAS-cog, interaction analyses between IGFBP-2 * APOE were significant ($\beta \pm SE$= -6.607$\pm$3.469, p=.020), as well as for MMSE ($\beta \pm SE$= 1.407$\pm$0.699, p=.045). These results indicate that among subjects with 1 or 2 APOE4 alleles, higher IGFBP-2 predicted better function on global assessments.

For memory, higher IGFBP2 was also related to higher scores on the RAVLT Delay ($\beta \pm SE$= 1.652$\pm$0.482, p=<.001). No main effects or interactions with APOE4 were shown with respect to a derived memory factor.

There were no IGFBP-2 by Baseline Diagnosis interactions. However, on an exploratory basis, when split by diagnosis, AD patients showed that IGFBP-2 was a significant predictor of worse scores on the constructional praxis portion of ADAS-cog, representing visual spatial abilities ($\beta \pm SE$= 1.189$\pm$0.580, p=.043). There were no significant associations between plasma IGFBP-2 and executive function.

AD CSF Biomarkers and Markers of Inflammation

Plasma IGFBP-2 was not associated with total tau, p-tau-181 or $A\beta_{1\text{-}42}$. However, higher levels of IGFBP-2 were related to higher expression of IL-6 receptor ($\beta \pm SE$ = 0.071$\pm$0.029, F=5.940, p=0.15) and lower C-peptide ($\beta \pm SE$=-0.156$\pm$0.043, F=13.289, p<.001). No significant differences were seen with respect to IGFBP-2 and APOE4 or Baseline Diagnosis interactions. Importantly, as noted in **Supplementary Text 4.1**, CSF IGFBP-2 was conversely related to all AD biomarkers but not peripheral inflammation.

Glucose, Insulin and HOMA-IR

Higher plasma IGFBP-2 was significantly associated with lower HOMA-IR (β=-.441, F=6.810, p=0.009) and insulin (β=-1.593, F=6.986, p=0.009), but not glucose (F=0.564, p=0.453). Subsequent IGFBP-2 * APOE interactions were non significant. 150 of the participants met fasting blood glucose requirements for pre-diabetes diagnosis (between 100 and 125 mg/dL), and 57 participants met fasting blood glucose requirements for diabetes diagnosis (>125 mg/dL). Both plasma and CSF IGFBP-2 were not predictive of whether or not a person met pre-diabetes or type 2 diabetes fasting blood glucose criteria. As noted in **Supplementary Text 4.1**, CSF IGFBP-2 was only related to higher glucose.

Regional Grey Matter Volume

Next, voxel-wise analysis was used to regress plasma IGFBP-2 concentrations against regional GM at baseline for 325 participants who had structural MRI data, demographic, and biological data. Higher plasma IGFBP-2 was related to less GM in a large cluster of voxels (k=128,608) across the right and left inferior parietal gyri, right frontal superior lobe, left postcentral gyrus, right cerebellum and right fusiform gyrus, with the maximum voxel located in the right frontal superior lobe (**Figure 4.2; Supplementary Table 4.2**). Smaller clusters included the cuneus, hippocampus, precuneus, and left and right superior prefrontal cortices. A similar pattern of results was found when regressing CSF IGFBP-2 against regional GM. Higher CSF IGFBP-2 was related to less GM primarily spanning the left and right amygdala, parahippocampus and hippocampus, among 186 participants who had structural MRI data, demographic, and biological data (**Supplementary Text 4.1**).

Regional FDG Metabolism

Higher plasma IGFBP-2 was related to less FDG glucose uptake in one cluster of 5419 voxels primarily spanning the superior dorsolateral prefrontal cortex, among 266 participants who had FDG data, demographic, and biological data (**Figure 4.3**).

Genetic Analysis of IGFBP-2

Linear regression in PLINK tested the additive genetic model of each SNP for association with cognitive scores while controlling for age, gender, and Baseline Diagnosis as covariates. Four variants in the IGFBP-2 locus on Chromosome 7 were nominally associated with worse cognitive function on the MMSE. Genotype distributions and odds ratios are listed in **Table 4.2.** RS4619 was nominally associated with MMSE (F=5.645, P=0.015), and the ADNI latent memory factor (F=6.115, P=0.013). Models comparing individual odds ratios and the average odds ratio between groups did not demonstrate significant differences between groups. Additionally, the relationship between these SNPs and baseline regional FDG and grey matter were assessed using voxel-wise analysis. Result maps indicated carriers of minor alleles in these SNPs showed less grey matter and FDG metabolism in a small region spanning the right frontal superior lobe. There was strong overlap between this result and what was found for IGFBP-2 gray matter and FDG result maps (**Figure 4.4**); however, the voxel wise result maps for individual SNPs marginally surpassed the ClusterSim statistical significance threshold of 462 voxels.

Discussion

In this study, we hypothesized that IGFBP-2 may serve as a useful biomarker for predicting AD outcomes. Importantly, higher IGFBP-2 corresponded to less grey matter in

AD-sensitive brain regions such as superior frontal gyrus and angular gyrus, and significantly less glucose uptake in the superior medial prefrontal cortex. Strikingly, for every point increase in IGFBP-2 levels, there was a 3 times increased risk of being diagnosed as MCI or AD when compared to CN. Curiously, however, higher IGFBP-2 corresponded to better cognitive performance in the RAVLT Delay, as well as global indices for APOE4 carriers only. These conflicting results may be due to a progression of insulin resistance, where the body is at first able to compensate for insulin resistance by releasing additional IGF to keep up with glucose demands of neuronal cells. Although much of glucose transport in the brain is insulin-independent via GLUT-1 and GLUT-3, insulin is necessary for GLUT-4 actions, which in rats has been detected in the cerebellum, olfactory bulb, and dentate gyrus of the hippocampus [28]. Although further research is necessary to translate these findings to humans, Craft et al. showed that intranasal insulin reduced progression of neuronal hypometabolism in individuals with MCI and AD, suggesting that insulin does play an important role in glucose uptake in the brain [29]. We hypothesize that central insulin resistance progresses to a point where GLUT-4 receptors are unable to respond to insulin and cells are delivered a suboptimal level of glucose. We suggest that the cognitive score results in our study are representative of the early compensatory response, which may in part be related to APOE4 status, while the grey matter, FDG, and diagnosis results are indicative of a post-compensatory state. In general, having one or more E4 alleles corresponded to higher IGFBP2 predicting better cognitive performance, whereas for non-APOE4s either no association or a detrimental pattern was observed, such as for ADAS-cog. Additionally, previous research has shown that individuals who are APOE4 positive have

lower expression of insulin degrading enzyme, thus potentially amplifying the early cognitive benefits of higher IGFBP-2 levels [30].

IGF-1 appears to be a key modulator of IGFBP-2 associations with AD outcomes. Because IGFBP is fundamental in regulating IGF bioavailability, the two proteins are intertwined in determining insulin expression and glucose levels in the periphery and brain, which this report illustrates at least in plasma. IGF-1 importantly determines glucose and lipid handling in the brain, myelin expression, and remodeling after neuronal injury [1]. Mice that under-expressed IGF-1 showed an accumulation of Aβ plaques, which were subsequently decreased after infusing the mice with IGF-1 [31].

To validate IGFBP-2 as a useful biomarker of AD and central IR, higher plasma IGFBP-2 corresponded with higher expression of plasma markers of inflammation including IL-6 receptor and C-peptide. Previous research has shown many correlations between inflammation in the brain and neuronal damage [32]. Obesity and insulin resistance are also associated with higher levels of inflammatory biomarkers [33].

We also compared and contrasted the utility of plasma IGFBP-2 to CSF IGFBP-2 for AD-related outcomes. CSF-based markers are often thought to provide better diagnostic accuracy in understanding the progression of diseases that cause cognitive impairment and dementia; however, collecting a CSF sample is more invasive. Our data indicate that when considering IGFBP-2, the plasma concentration was a better predictor of brain structure and cognition, while only CSF concentrations reflected CSF amyloid and tau. Future studies should take into account the differing clinical utility of blood-based and CSF biomarkers against the practicality of sample collection, in addition to their predictive utility of disease

progression. Improving how biomarkers are used in clinical trials will likely provide more precise diagnoses to patients with AD.

Subsequently, we examined how IGFBP SNPs influenced AD-related outcomes. Since population stratification can result in erroneous genetic associations, we restricted our analyses to only subjects of Northern and Western European heritage. While the exact mechanism has yet to be revealed, our data show that genetic variation in IGFBP-related genes modified cognitive decline. This may be in part due to modifying IGF-1 regulation of glucose in the brain, ultimately increasing neuronal vulnerability to induce cognitive decline. Genetic variation negatively affecting IGFBP-2 signaling may decrease the protective effects that circulating IGFBP-2 exerts on cognition described above, whereby these SNPs may predispose individuals to develop AD. Previous large-scale genome-wide association studies (GWAS) examining genetic association with AD have not implicated SNPs at IGFBP loci because these variants commonly do not surpass genome wide significance of P < 1X10^-8. Performing targeted genomic association analysis in combination with neuroimaging analysis provides a unified approach to understand how genomic variation may contribute to AD symptoms.

The limitations of this study should be addressed. This study included a modest sample size, where the exploratory nature of this study design would benefit from a larger sample. It is worth noting that these analyses did not show any significant correlations between plasma or CSF IGFBP-2 and MCI conversion. However, a larger sample size of individuals with MCI may be needed to see if IGFBP-2 can predict if an individual with MCI converts to AD. Furthermore, since this is a cross-sectional study using baseline data from

the ADNI cohort, it was beyond the scope of the project to determine the causal effects between IGFBP-2 on longitudinal CSF biomarkers and disease progression. Additionally, we could not correlate IGFBP-2 with all of the disease state associations throughout the literature such as gastric cancer or eating disorders because this was beyond the scope of ADNI's mission. Like all genetic studies, these results should be validated using additional independent and larger cohorts. Finally, other unrecognized cellular pathways, not related to neurodegeneration, may be disrupted that influence IGF-1 signaling and AD progression.

Conclusion

This study provides evidence that plasma IGFBP-2 is associated with AD risk, brain atrophy and less glucose metabolism in regions sub-serving memory and global cognitive function. We also demonstrated that plasma IGFBP-2 served as a more comprehensive predictor of AD-related outcomes than CSF IGFBP-2. These results illustrate the potential that integration of genomic, biologic and neuroimaging data may lead to identification of novel targets for treatment of AD while improving the overall understanding of potential mechanisms underlying the pathophysiology of AD. In conclusion, insulin signaling mechanisms such as IGFBP-2 may be important for future therapeutics to target via genetic modification or regulating circulating IGFBP-2 to delay the onset of AD by improving neural metabolism and cognitive function.

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Authors' Contributions

K.E.M. conducted data analyses and prepared this manuscript. J.L.W. conducted data analyses and prepared this manuscript. V.A. provided analysis guidance and edited manuscript. A.K. provided analysis guidance and edited manuscript. A.A.W. conducted data analyses, provided analysis guidance and prepared and edited this manuscript.

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Tables and Figures

Table 4.1. Demographic Data for Subjects with Plasma IGFBP-2

	CN (N=58)	MCI (N=396)	AD (N=112)
Age	76.27 ± 5.73	74.91 ± 7.49	75.23 ± 8.39
Education (years)	15.67 ± 2.78	15.64 ± 3.0	15.1 ± 3.2
Sex % Female	48	35	42
% APOE 4 carriers * **	9%	49%	64%
Plasma IGFBP2 (ng/mL) * **	1.88 ± 0.20	1.99 ± 0.23	1.91 ± 0.12
C Peptide (ng/mL)	0.37 ± 0.21	0.39 ± 0.21	0.36 ± 0.18
IL-6 Receptor (ng/mL)*	1.51 ± 0.12	1.46 ± 0.14	1.47 ± 0.11
Glucose (mg/dL)	102.33 ± 23.47	101.42 ± 27.25	99.06 ± 22.48
Insulin (uIU/mL)	3.00 ± 2.34	2.64 ± 2.73	2.38 ± 1.43
HOMA-IR	0.82 ± 0.80	0.68 ± 0.75	0.59 ± 0.39
CSF Total Tau (pg/mL)* **	63.62 ± 21.76	102.66 ± 59.81	120.30 ± 56.33
Ptau (pg/mL)* **	21.07 ± 8.43	36.19 ± 19.28	41.92 ± 19.90
Abeta 42 (pg/mL)* **	250.85 ± 21.08	163.97 ± 53.15	142.69 ± 39.15
CDR-SOB* **	0.03 ± 0.11	1.61 ± 0.88	4.31 ± 1.61
MMSE* **	28.93 ± 1.16	27.02 ± 1.78	23.59 ± 1.96
Immediate RAVLT* **	40.98 ± 7.19	30.73 ± 9.05	23.54 ± 7.42
Delayed RAVLT*	3.73 ± 3.13	4.72 ± 2.25	4.47 ± 1.94
ADAS-COG* **	6.37 ± 2.76	11.52 ± 4.38	18.27 ± 6.37
ADNI_MEM Score* **	0.46 ± 0.72	0.03 ± 1.04	-0.35 ± 0.85
Executive Function* **	0.71 ± 0.58	-0.03 ± 0.78	-0.93 ± 0.83

Values are mean ± SD, * indicates ANOVA $p<0.05$; **indicates $p<0.01$; *** indicates ANOVA or chi-square $p<0.001$. Chi-square analyses were conducted to examine differences between gender and APOE4 status. The ADNI memory factor values are Z-scored with mean 0 and a standard deviation of 1, based on the 810 subjects with baseline memory data [20].

Table 4.2. Association of IGFBP-2 SNPs with Mini-Mental Status Exam (MMSE) scores and the ADNI Memory composite score

	MMSE				ADNI Memory Factor			
SNP	**Common Allele**	**Effect Allele**	**Beta**	**Beta P value**	**Common Allele**	**Effect Allele**	**Beta**	**Beta P value**
rs4619 AA vs GA + GG	29.1 ± 1.55	24.9 ± 2.02	-0.32	P = 0.089	1.01 ± 0.59	−0.14 ± 0.64	-0.12	P = 0.047
rs138105891 AA vs GA + GG	28.5 ± 1.31	25.4 ± 0.57	-0.21	P = 0.044	0.95 ± 0.44	0.33 ± 0.39	-0.30	P = 0.068
rs41258845 CC vs GC + GG	29.2 ± 1.17	25.6 ± 1.02	-0.34	P = 0.199	1.03 ± 0.67	−0.04 ± 0.77	-0.11	P = 0.041
rs1065782 GG vs GA + AA	27.2 ± 1.75	24.5 ± 1.09	-0.17	P = 0.441	1.10 ± 0.48	−0.02 ± 0.58	-0.22	P = 0.052

Beta values represent the difference in the predicted value of either MMSE or the ADNI memory factor based on an increase from no risk allele, one risk allele, or two risk alleles.

Supplementary Table 4.1: Demographic Data for Subjects with CSF IGFBP-2

	CN (N=45)	MCI (N=134)	AD (N=66)
Age	76.7 ± 5.9	75.7 ± 7.4	76.0 ± 7.5
Education	15.5 ± 2.9	16.0 ± 3.0	15.1 ± 3.0
Sex % Female*	49%	31%	44%
% APOE 4 carriers ***	9%	49%	67%
CSF IGFBP2 Peptide	23.77 ± 0.3 0	23.78 ± 0.31	23.82 ± 0.37
ADNI_MEM Score***	0.83 ± 0.49	-0.15 ± 0.53	-0.84 ± 0.58

Values are mean ± SD, * Indicates ANOVA P<0.05, *** = P<0.001. The ADNI memory factor values are Z-scored with mean 0 and a standard deviation of 1, based on the 810 subjects with baseline memory data.

Supplementary Table 4.2: Main effects of serum IGFBP-2 on regional grey matter volume

Location	T value	X, Y, Z	Cluster size (voxels)
Frontal Superior (R)	5.27	-15, 31, 55	128608
Angular (R, L)	5.25	34, -58, 51	
Parietal Inferior (L)	5.07	-54, -27, 45	
Frontal Middle (R)	5.04	44, 15, 46	
Precentral Gyrus (R)	4.96	48, 6, 38	
Supplemental Motor Area (L)	4.88	-7, 14, 67	
Parahippocampal (R)	4.81	21, 4, -34	
Precuneus (R)	4.77	3, -58, 51	
Hippocampus (L)	4.59	-28, -39, 4	
Anterior Cingulum	4.51	-5, 39, 4	
Amygdala (L)	4.43	-22, -3, -13	
Parahippocampus (R)	4.33	17, -32, -13	
Frontal Inferior (R)	4.32	40, 8, 28	
Cuneus (L)	4.21	-3, -81, 34	
Postcentral Gyrus (R)	4.14	36, -34, 50	
Frontal Superior (L)	4.21	-12, 57, 26	1206
Frontal Superior Medial (L)	4.73	-4, 28, 46	

This table depicts regions where all subjects had less predicted gray matter volume as IGFBP-2 increased. The highest *t* value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest *t* value in those areas is indicated. Coordinates are in MNI atlas space. Brains are oriented in neurological space.

L left hemisphere, *R* right hemisphere

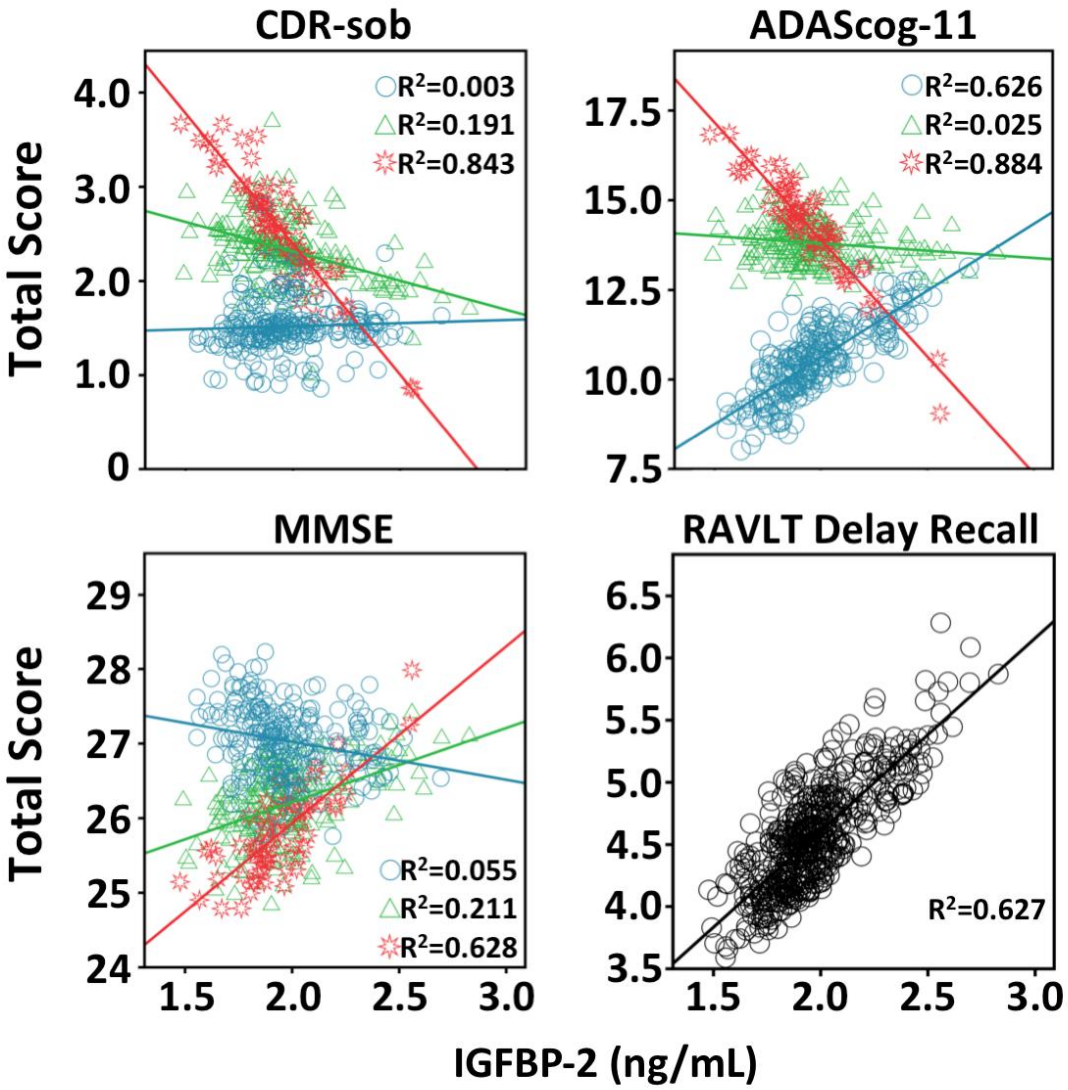

Figure 4.1: Cognitive scores and IGFBP-2 analyses. The association between plasma IGFBP-2 and the Clinical Dementia Rating sum of boxes (CDR-sob), Mini-Mental Status Exam (MMSE), Rey Auditory Verbal Learning Test (RAVLT), AD Assessment Schedule - Cognition (ADAS-Cog). “Blue circle” indicates 0 APOE4 alleles, “green triangle” indicate 1 APOE4 allele, and “red star” indicates 2 APOE4 alleles.

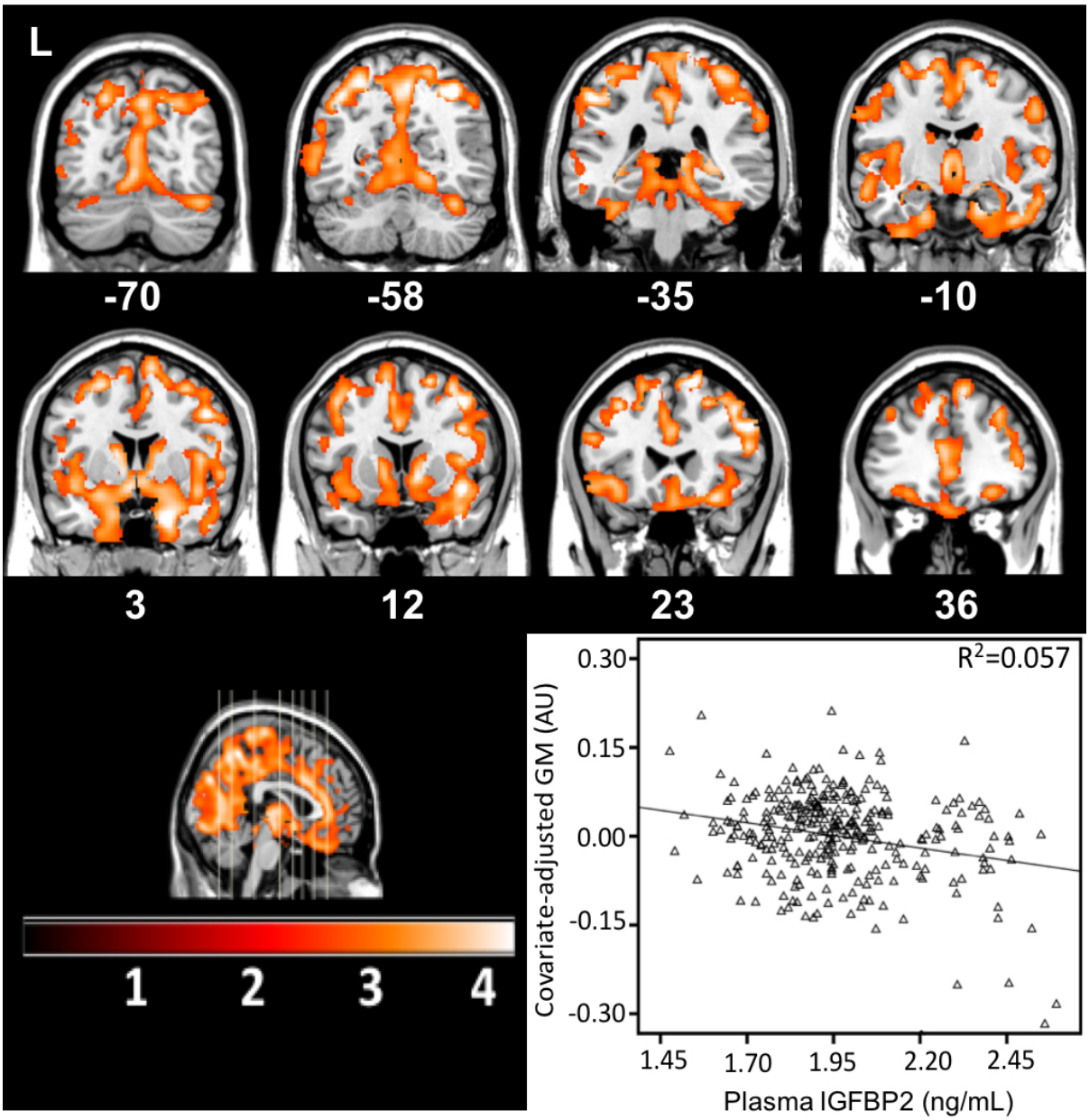

Figure 4.2: Brain areas showing less grey matter corresponding to higher plasma IGFBP-2.

The graph depicts the relationship at a sub-maximal voxel in dorsal prefrontal cortex.

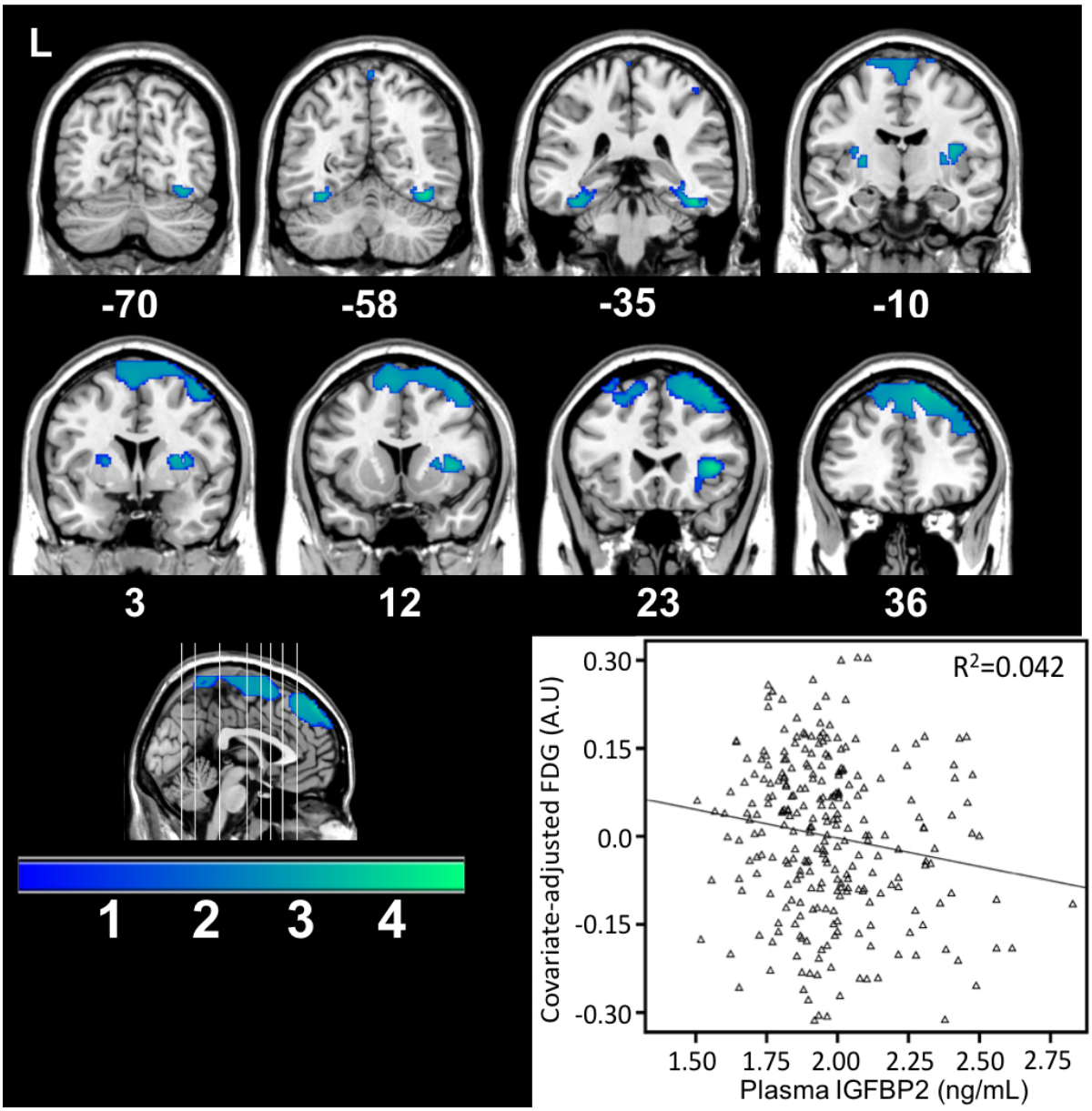

Figure 4.3: Brain areas showing less FDG metabolism corresponding to increased plasma IGFBP-2. The graph depicts the relationship at a sub-maximal voxel in the dorsal prefrontal cortex.

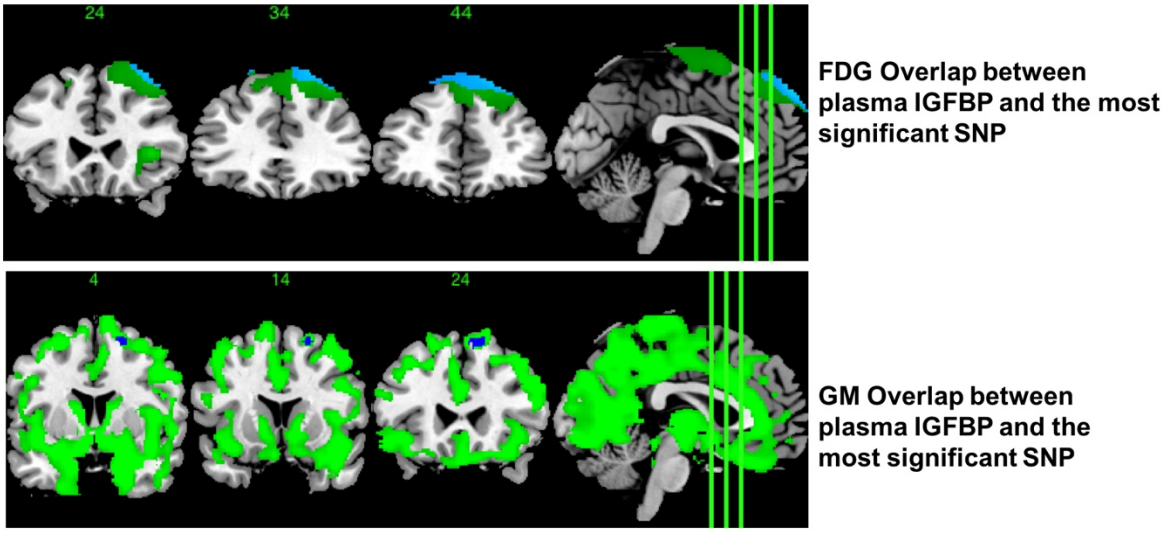

Figure 4.4: Brain areas showing overlap between either the GM or FDG and IGFBP2 result maps and the result map of the strongest SNP association for GM or FDG.

Supplementary Text 4.1 – CSF IGFBP 2 and Outcomes

Global, Memory, and Visual Spatial Indices

No significant differences were shown with regards to cognitive indices and CSF IGFBP-2. However, there was a significant negative CSF IGFBP-2 * Age interaction with executive function (F= 6.417, p=0.012), indicating that among older subjects, higher CSF IGFBP-2 was related to worse performance.

For visual spatial assessment, there was an APOE Status * IGFBP-2 interaction for the clock drawing test total score (F=4.912, p=.008) and the construction stimulus score of the MMSE exam (F=4.653, p=.010). Among APOE4 carriers, higher IGFBP-2 was related to lower scores. Similarly, for the clock drawing test, an Age * IGFBP-2 interaction (F=5.806, p=0.017) showed that among older subjects, higher IGFBP-2 predicted a lower total score.

AD CSF Biomarkers and Markers of Inflammation

Higher CSF IGFBP-2 concentrations were related to higher levels of CSF tau (F=17.134, p<.001), phosphorylated tau (F=4.121, p=.044), and CSF Aβ1-42 (F=5.265, p=.023). CSF IGFBP-2 was not related to any of the biomarkers of inflammation.

Glucose, Insulin and HOMA-IR

CSF IGFBP-2 was not associated with HOMA-IR (F=0.127, P=0.143) or with insulin (F=0.753, P=0.231); however, it was significantly related to higher glucose (F=9.554, P=0.002).

Regional Grey Matter Volume

CSF IGFBP-2 was regressed against regional GM at baseline using VBM. Higher CSF IGFBP-2 was related to less GM in two clusters (9917 and 15455 voxels) spanning areas

similar to the plasma results, as well as the left and right inferior parietal, cerebellum and right fusiform gyrus, with the maximum voxel located in the right frontal superior lobe.

Regional FDG Uptake

CSF IGFBP-2 showed no significant associations with FDG metabolism among the 126 participants who had FDG data, demographic, and biological data.

CHAPTER 5. IS CSF SOD1 A BIOMARKER OF TAU BUT NOT AMYLOID INDUCED NEURODEGENERATION IN ALZHEIMER'S DISEASE?

Modified from a manuscript published in Antioxidants and Redox Signaling

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Abstract

Copper/zinc superoxide dismutase (SOD1) scavenges free radicals that may otherwise damage brain parenchyma. Impaired SOD1 activity drives AD neuropathology in animal models and post-mortem AD brains. Yet, it is unknown how CSF SOD1 is related in vivo to AD-relevant cognitive, neuroimaging, and CSF neurotoxic factors, and what potential

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

mechanisms underlie these associations. We found that higher CSF SOD1 correlated with better global cognition scores, yet less gray matter (GM) and glucose metabolism in AD-sensitive parietal and frontal regions. Higher CSF SOD1 was also associated with more CSF total tau and phosphorylated tau-181, but not beta-amyloid (Aβ) 1-42. Through mediation analyses, higher total tau largely mitigated higher CSF SOD1 and better global cognition associations, and it fully accounted for less predicted regional GM but not glucose metabolism. Among participants who developed AD over 2 years or had AD at baseline, higher CSF SOD1 was initially related to more regional GM. This association became non-significant with full mediation via higher CSF total tau, whereby higher CSF SOD1 predicted more total tau and in turn less GM. Our observations lead to the hypothesis that SOD1 antioxidation reflects tau but not amyloid accumulation, which may lead to prooxidant-based neurodegeneration and cognitive dysfunction.

Introduction

Oxidative stress is thought to play a major role in the pathophysiology of AD. Particularly, brain proteins and lipids are susceptible to oxidative damage. It is unclear if the oxidative damage seen in AD is a result of the disease process or causes neurological decline. SOD is a ubiquitous enzyme that protects against ROS formed during aerobic respiration(9). Specifically, SOD is an antioxidant enzyme that catalyzes the oxidation-reduction reaction of the superoxide anion ($O_2^{\bullet -}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). SOD consists of three isoenzymes: copper/zinc SOD, or SOD1, localized predominantly to the cytosol; manganese SOD, or SOD2, within mitochondria; and

extracellular SOD, or SOD3. Changes in SOD levels can cause excess ROS in cells, leading to altered intracellular and mitochondrial metabolism, as well as DNA and vascular damage.

SOD1 has been examined in AD for its role in preserving neural integrity and memory performance. Increased SOD1 levels are seen in the pyramidal neurons of AD patients compared to controls(3). For hippocampal cells of AD patients and related transgenic models, SOD1 overexpression downregulates Aβ-induced oxidative stress, using a non-oxidized versus oxidized glutathione ratio as a marker(2). Further, co-expression of SOD1 in APP transgenic mice blocks endothelial dysfunction that gives rise to hypoperfusion often seen in AD(5). SOD1-deficient Tg2576 mice, meanwhile, show increased Aβ plaque formation, neuroinflammation, tau phosphorylation, and memory decline(6).

In this study, CSF SOD1 data collected by the Alzheimer's Disease Neuroimaging Initiative (ADNI) in 287 aged adults was examined with respect to CSF $A\beta_{1-42}$ and tau pathology markers, brain atrophy and glucose uptake, and cognitive performance.

Innovation

Despite SOD1's protective role for memory and neuropathology seen in post-mortem AD brains and AD transgenic models, it is unknown how SOD1 is related in vivo to cognition, brain, and CSF toxic marker outcomes across the AD spectrum. Via mediation, we found that higher CSF total tau and to a lesser extent phosphorylated tau, but not CSF $A\beta_{1-42}$, largely mitigated "beneficial" global cognition associations. Total tau fully mediated SOD1 predicted atrophy in AD-sensitive regions. We hypothesize that CSF SOD1 is an in vivo biomarker of tau but not amyloid related neurodegeneration in AD, where tau's causal role remains unclear.

Results

Data Summary

Clinical and demographic data on 287 ADNI participants are presented in **Table 5.1**. There were no differences based on years of education, age, or CSF SOD levels at baseline between CU, MCI or AD subjects. As expected for this ADNI sub-population, the percentage of APOE4 carriers increased stepwise from CU to AD. There was also significantly fewer women in the MCI and AD relative to CU groups.

Global Cognition and Memory

Broadly, we observed that higher CSF SOD1 was related to "better" scores for global cognition but not sub-domain tests. Specifically, for CDR-sob, higher CSF SOD-1 was significantly associated with better scores ($\beta \pm SE = -0.625 \pm 0.285$, $p<0.05$). Similarly, SOD1 was related to better ADAS-cog11 ($\beta \pm SE = -2.768 \pm 1.033$, $p<0.01$) and MMSE scores ($\beta \pm SE = 0.973 \pm 0.397$, $p<0.05$). SOD1 main effects, or interactions with APOE4 status or baseline clinical diagnosis, were non-significant for memory and executive function factors, as well as RAVLT indices.

AD CSF Biomarkers

CSF SOD1 was significantly related to higher levels of total tau ($\beta \pm SE = 81.666 \pm 5.986$, $p<.001$) and p-tau-181 ($\beta \pm SE = 18.440 \pm 2.253$, $p<.001$) **(Figure 5.1)**. No significant differences were seen with respect to SOD1 and CSF $A\beta_{1-42}$.

Regional FDG Metabolism

Next, voxel-wise analysis was used to regress CSF SOD1 levels against regional FDG uptake, an index of glucose metabolism, at baseline. Higher CSF SOD1 was significantly

associated with less FDG (p<.05, FWE corrected) in a large cluster with the maximum in left supplementary motor area (**Figure 5.2**, "blue" cluster**; Table 5.2**). This cluster bilaterally spanned superior medial frontal gyrus, mid- and posterior cingulate gyri, pre- and post-central gyri, precuneus, lobulo-limbic transitional gyrus, as well as left angular gyrus. Smaller clusters encompassed anterior cerebellum or pre- and post-central gyri.

Regional Grey Matter Volume

Voxel-wise analysis was also used to examine how CSF SOD1 concentrations were associated with regional GM at baseline. Higher CSF SOD1 was related to less GM (p<.05, FWE corrected) in a large cluster (**Figure 5.2,** "green" cluster; **Table 5.3**), with the maximally significant voxel in left supplementary motor area. This cluster bilaterally encompassed precuneus and pre- and post-central gyri, as well as left lobulo-limbic transitional gyrus/paracentral lobule, superior parietal gyrus, and superior frontal gyrus (**Figure 5.2**; **Table 5.3**).

Preacher-Hayes Mediation of SOD1 for Memory, GM, and FDG-PET Outcomes

Lastly, Preacher-Hayes mediation analyses were used to separately determine if CSF total tau, p-tau-181, or $A\beta_{1-42}$, acted as mediator for the associations between SOD1 and global cognition, GM, and FDG outcomes. In summary, CSF total tau or to a lesser extent p-tau-181, but not CSF $A\beta_{1-42}$, partially or fully mediated SOD1 associations.

For the higher SOD1 and better MMSE score association (direct effect β±SE = 2.980 ± 0.441, p<.001), higher total tau acted as a partial mediator, decreasing the statistical effect of SOD1 on MMSE by 67.6% (indirect effect β±SE = -2.014±0.358, p<.01) (**Figure 5.3A**). In a separate model, P-tau-181 also showed significant, albeit weaker partial mediation of the

SOD1 and MMSE association, decreasing the statistical effect by 51.5% (direct effect β±SE = 2.002 ± 0.407, p<.001; indirect effect β±SE = -1.028 ± 0.237, p<.01). Similar associations were seen for CDR-sob and ADAS-cog11.

For the higher CSF SOD1 and less regional GM association, we focused on precuneus because hypometabolism and atrophy there are imaging markers of progression from preclinical AD to AD. As shown in **Figure 5.1**, SOD1 originally predicted less precuneus GM to a moderate degree (β±SE = -0.064±0.0142, p<.001). CSF total tau fully mediated this relationship (indirect effect: β±SE = -0.035 ± 0.015, p<.05) **(Figure 5.3B)**, where the SOD1-GM direct effect was non-significant (β±SE = -0.047±0.024, p>.05)**.** In a separate model, P-tau-181 partially mediated SOD1 and precuneus GM associations, increasing the degree of association by 35% (direct effect: β±SE = -0.049±0.016, p<.05; indirect effect: β±SE = -0.017±0.008, p<.05). Total tau and p-tau-181 did not mediate associations between higher CSF SOD1 and less baseline regional FDG uptake.

To explore if AD onset and progression drove these GM mediation results, we re-ran models only with MCI participants who converted to AD within 24 months and participants classified as AD at baseline. As shown in **Figure 5.4**, higher CSF SOD1 was related to more regional GM in precuneus (direct effect: β±SE = 0.106 ± 0.034, p<.01). Higher total tau levels fully mediated this association via suppression, decreasing the statistical effect on GM by 90% (indirect effect: β ± SE = -0.094±0.027, p<.01) and rendering the total effect association between CSF SOD1 and more regional GM non-significant. Recall that for voxel-wise analyses including all participants (see **Figure 5.3B**), higher SOD-1 predicted less GM that was also fully mediated by higher CSF total tau.

Discussion

Progressive pro-oxidant damage has been associated with AD hallmarks such as tau and amyloid neuropathology, memory decline, and less neural integrity in AD-sensitive regions. In this study, we observed across the AD spectrum that more CSF SOD1 was related to more CSF tau species but not $A\beta_{1-42}$. CSF total tau via mediation suppressed "beneficial" SOD1 associations with cognition and fully accounted for less predicted GM, but not glucose uptake, in AD-sensitive parietal and frontal regions. Even so, this leads to the further question of whether total tau induces pro-oxidation or reflects a specific pro-oxidative process separate from $A\beta_{1-42}$.

In AD rodent models, higher SOD1 expression generally appears to induce protection in brain (1,5,6) and preserve cognition (4). We observed that higher CSF SOD1 corresponded to better global cognitive function based on CDR-sob, ADAS-cog11 and MMSE in all participants. This is consistent with a study among aged mice that overexpressed extracellular SOD (i.e., SOD1), which showed improved motor learning and decreased age-related decline in spatial memory as compared to controls(4). The authors also illustrated an improvement in long-term potentiation, a molecular process underlying learning and memory, in the mice overexpressing extracellular SOD. While our observations are correlational and mediation with this data cannot be used to make causal interpretations, it was nonetheless interesting that higher total tau, but not CSF $A\beta_{1-42}$, reflected drastic mitigation but not erasure of CSF SOD1 associations with global cognition.

Here, regardless of clinical diagnosis, higher SOD1 levels across all participants were strongly correlated with more CSF total tau and p-tau-181 levels but not $A\beta_{1-42}$. This led to

our hypothesis that progressive neurodegeneration reflecting more tau deposition might induce more SOD1 to minimize free radical damage. These findings parallel Winer and colleagues, who showed a significant positive correlation between CSF SOD1 levels and both tau and phosphorylated tau in aged adults with AD (8). They also observed weak CSF SOD1 associations with CSF $A\beta_{1-42}$ across all diagnostic groups and no associations among any of the sub-groups (8), which is in line with our observing no such relationships in this study. We hypothesize that SOD1 activity may reduce tau-related neurodegeneration in areas affected later in the disease, where other antioxidant factors may regulate pro-oxidant damage reflecting amyloidosis. Alternatively, since tau is indicative of neuronal injury, and oxidative stress can cause cell death, tau and SOD-1 may track neurodegeneration during AD onset and progression better than $A\beta_{1-42}$.

Indeed, for regional GM and FDG metabolism, CSF SOD1 was related to less GM and glucose metabolism in precuneus, posterior cingulate gyrus, paracentral lobule, and frontal gyri. Our mediation analyses suggest that tau accumulation reflecting progressive pro-oxidant damage may affect atrophy in these areas, which in part distinguish AD onset and progression. Indeed, CSF SOD1 no longer significantly predicted more GM in participants with AD onset or AD at baseline after total tau mediation. It is our hypothesis that SOD production may thus be futile in controlling free radical levels once a participant is on a trajectory for AD onset and progression. This phenomenon may extend beyond neural parenchyma. For example, Erythrocyte SOD activity was significantly higher in a group of 27 AD participants compared to controls; the SOD levels in the AD and control participants were also positively correlated with serum malondialdehyde levels, which is a marker of

oxidative stress, suggesting that the body is producing SOD in an attempt to reduce oxidative stress levels(8).

We should note several limitations with this study. First, all analyses were correlational, and while mediation analyses suggested more total tau largely drove SOD1 associations, it remains unclear if tau accumulation induces redox imbalance or serves an antioxidant function to reduce pro-oxidative damage. Therefore, experimental paradigms are needed to test if AD human tau neurons in transplanted in AD or control rodent parietal and frontal regions induce pro- or anti-oxidation, and how CSF SOD1 application affects neurite integrity with or without tau present. Dietary recall was also not collected in ADNI participants, which would have been helpful to assess the link between diets that induce oxidative stress and SOD1 expression in the brain. Participant CSF samples were only assayed at baseline, so we were unable to longitudinally examine CSF SOD1 and variation over time in AD outcomes. Lastly, plasma SOD1 that was assayed in ADNI1 did not pass quality control measures, which did not allow us to see if similar associations occurred in the periphery.

In summary, we observed that CSF SOD1 has strong associations with total tau and p-tau-181 in CSF, global cognition, and regional GM and glucose metabolism, all of which have been linked with AD etiopathogenesis. We observed that CSF SOD1 is associated with better global cognition, yet less GM and glucose uptake in parietal and frontal areas that are sensitive to AD-related changes. We noted that higher total tau appears to partially or fully mediate these associations, nearly mitigating the statistical effect of more SOD1 on better MMSE scores and less regional GM volume. Because of these novel findings, we

hypothesize that CSF SOD1 may simultaneously be related to the degree of AD-related neurodegeneration seen in the brain specific to tau accumulation, but also reflect the degree of free radical scavenging as a compensatory mechanism. Future work should examine if CSF SOD1 levels are related to regional tau deposition and track progressive accumulation across the AD spectrum; additionally, forthcoming research should determine the utility of peripheral SOD1 as a biomarker for AD outcomes.

Notes

Participants

Data from middle-aged to aged adults were obtained from the ADNI database (http://adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of MCI and early AD. For up-to-date information, see http://www.adni-info.org. Written informed consent was obtained from all ADNI participants at their respective ADNI sites. The ADNI protocol was approved by site-specific institutional review boards. Baseline CSF data for SOD1 was available for 287 subjects: 86 CU individuals, 135 MCI individuals, and 66 individuals with AD.

Participants with MCI met the following diagnostic criteria: 1) memory complaint identified by the participant or their study partner; 2) abnormal memory as assessed by the Logical Memory II subscale from the Wechsler Memory Scale- Revised; 3) Mini-Mental State Exam (MMSE) score between 24 and 30; 4) Clinical dementia rating of 0.5; 5) Deficits not

severe enough for the participant to be diagnosed with Alzheimer's disease by the site physician at screening. Participants with AD met similar criteria, however, were required to have an MMSE score between 20 and 26, a clinical dementia rating of 0.5 or 1.0, and NINCDS/ADRDA criteria for probable AD.

Mass Spectrometry and SOD1

Data were downloaded from the Biomarkers Consortium CSF Proteomics MRM dataset. The ADNI Biomarkers Consortium Project investigated the extent to which selected peptides, measured with mass spectrometry, could discriminate among disease states. Briefly, Multiple Reaction Monitoring-MS (MRMMS) was used for targeted quantitation of 567 peptides representing 221 proteins in a single run (Caprion Proteome Inc., Montreal, QC, Canada). Analyses for this report focused on cytosolic SOD1 levels, which were assayed in the CSF multiplex panel as reflected by the peptide GDGPVQGIINFEQK.

APOE Genotype

The ADNI Biomarker Core at the University of Pennsylvania conducted APOE ε4 genotyping. We characterized participants as having zero APOE4 alleles, one APOE4 allele, or two APOE ε4 alleles. Analyses did not differ when discriminating APOE4 carriage vs. non-carriage.

Amyloid and Tau CSF Biomarkers

CSF sample collection, processing, and quality control of p-tau-181, total tau, and $A\beta_{1-42}$ are described in the ADNI1 protocol manual (http://adni.loni.usc.edu/). Briefly, CSF samples were analyzed using INNO-BIA Alz Bio3 immunoassay reagents with the Luminex

platform. Statistical modeling was completed to confirm reliability of results across seven laboratories before implementation with ADNI.

Neuropsychological Assessment

ADNI utilizes an extensive battery of assessments to examine cognitive functioning with particular emphasis on domains relevant to AD. A full description is available at http://www.adni-info.org/Scientists/CognitiveTesting.aspx. All subjects underwent clinical and neuropsychological assessment at the time of scan acquisition. Neuropsychological assessments included: The Clinical Dementia Rating sum of boxes (CDR-sob), Mini-Mental Status Exam (MMSE), Auditory Verbal Learning Test (RAVLT) and AD Assessment Schedule - Cognition (ADAS-Cog). A composite memory score encompassing the RAVLT, ADAS-COG, MMSE, and Logical Memory assessments was also utilized. This composite memory score was used in formal analyses to represent global memory among subjects.

Magnetic Resonance Imaging (MRI) Acquisition and Pre-Processing

T1-weighted MRI scans were acquired within 10-14 days of the screening visit following a back-to-back 3D magnetization prepared rapid gradient echo (MP-RAGE) scanning protocol described in the ADNI MRI Technical Procedures Manual (https://adni.loni.usc.edu). The SPM12 “New Segmentation” tool was used to extract modulated gray matter (GM) volume maps. Maps were smoothed with an 8mm Gaussian kernel and then used for all voxel-wise analyses.

FDG-PET

Briefly, 185 MBq of [18-153-F]-FDG was injected intravenously. After 30 minutes, six 5-minute frames were acquired. Frames of each baseline image series were co-registered to

the first frame and combined into dynamic image sets. Each set was averaged, reoriented to a standard 160 x 160 x 96 voxel spatial matrix of resliced 1.5 mm^3 voxels, normalized for intensity, and smoothed with an 8 mm FWHM kernel. In order to derive the standardized uptake value ratio (SUVR), pixel intensity was normalized according to the pons since it demonstrates preserved glucose metabolism in AD. Normalization to the pons removed inter-individual tracer metabolism variability. The Montreal Neurological Institute (MNI) template space was used to spatially normalize images using SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/).

Statistical Analysis

All analyses were conducted using SPSS 25 (IBM Corp., Armonk, NY) or SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). For non-MRI or FDG analyses, linear mixed regression models tested the main effects of CSF SOD1 on outcomes including neuropsychological performance and AD CSF biomarkers including $A\beta_{1-42}$, total tau and p-tau-181. Main effects and interactions with APOE4 allele load were separately tested in a single model. Covariates included age at baseline, BMI, sex, and baseline clinical diagnosis. Participant years of education was also included as a covariate for models with cognitive tests as an outcome of interest. Baseline diagnosis was not used as a covariate for models with cognitive tests as an outcome of interest to avoid multicollinearity. Preacher Hayes mediation analyses were conducted in SPSS 25 using PROCESS v3.3. Non-significant covariates were removed from final models for model parsimony.

For voxel-wise analysis, 2nd-level mixed models tested main effects of SOD1 on regional GM volume and FDG, controlling for age, sex, BMI, APOE4 status, and baseline

clinical diagnosis. Voxel and cluster statistical thresholds were set at p<.005 (uncorrected) and p<.05 (FWE corrected) respectively. Results were considered significant at the cluster level. As described previously(7), in order to further reduce type 1 error, we utilized a GM threshold of 0.2 to ensure that voxels with <20% likelihood of being GM were not analyzed. For GM, Monte Carlo simulations in ClusterSim (http://afni.nimh.nih.gov/afni/doc/manual/3dClustSim) were used to estimate that 462 contiguous voxels were needed for such a cluster to occur at FWE $p < 0.05$. For FDG voxel-wise analyses, Monte Carlo simulations were used to estimate that 224 contiguous voxels were needed for such a cluster to occur at FWE $p < 0.05$.

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Scale Diagnostics, LLC.; NeuroRx Research; Neurotrack Technologies; Novartis Pharmaceuticals Corporation; Pfizer Inc.; Piramal Imaging; Servier; Takeda Pharmaceutical Company; and Transition Therapeutics. The Canadian Institutes of Health Research is providing funds to support ADNI clinical sites in Canada. Private sector contributions are facilitated by the Foundation for the National Institutes of Health (www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Therapeutic Research Institute at the University of Southern California. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of Southern California.

Authors' Contributions

K.E.M. conducted analyses and prepared manuscript. B.E.C. conducted analyses and prepared manuscript. A.P. conducted analyses and edited manuscript. B.K. conducted analyses and edited manuscript. C.P. provided analysis suggestions and edited manuscript. V.A. provided analysis suggestions and edited manuscript. A.K. provided analysis suggestions and edited manuscript. A.A.W. oversaw project design and analyses and prepared and edited manuscript.

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Tables and Figures

Table 5.1: Demographic data for ADNI subjects with CSF SOD1

	CU (N=86)	MCI (N=135)	AD (N=66)
Age (years)	75.70 ± 5.54	74.67 ± 7.32	75.04 ± 7.62
Education (years)	15.64 ± 2.97	16.02 ± 2.97	15.06 ± 2.93
Sex (% female)*	48.8%	32.6%	43.9%
BMI (kg/m^2)	26.54 ± 3.84	25.98 ± 3.77	26.36 ± 4.28
APOE4 carriage (%)***	24.4%	52.6%	71.2%
CSF SOD (AU)	21.46 ± 0.34	21.49 ± 0.37	21.42 ± 0.39
CDR-sob (score)***	0.02 ± 0.11	1.54 ± 0.86	4.37 ± 1.54
ADAS-cog11 (score)***	6.05 ± 2.90	11.73 ± 4.33	18.86 ± 6.73
EF factor (Z-score)***	0.66 ± 0.62	-0.08 ± 0.73	-1.02 ± 0.81
Memory factor (Z-score)***	0.98 ± 0.50	-0.15 ± 0.57	-0.90 ± 0.56
MMSE (score)***	29.05 ± 1.02	26.92 ± 1.74	23.50 ± 1.83

Values are mean ± SD unless otherwise stated. *,***= Chi-square $p < .05, < .001$.

Table 5.2: Negative main effects of SOD on FDG metabolism

Location	T value	X, Y, Z	Cluster size (voxels)
Mid Cingulate Gyrus (R)	5.31	4, -2, 44	5214
Precuneus (R)	4.72	2, -48, 44	
Mid Cingulate Gyrus (R)	4.25	4, -20, 48	
Cerebellum	3.28	-6, -58, -14	263
Cerebellum	3.18	-8, -64, -22	
Precentral Gyrus (R)	3.00	34, -16, 68	282
Postcentral Gyrus (R)	2.83	38, -36, 70	

This table depicts regions where all subjects had less predicted FDG metabolism as a function of more CSF SOD1. The highest *t* value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest sub-maxima *t* values in those areas are indicated. Coordinates are in MNI atlas space. Brains are oriented in neurological space.

L left hemisphere, *R* right hemisphere

Table 5.3: Negative main effects of SOD on regional grey matter volume

Location	T value	X, Y, Z	Cluster size (voxels)
Supplementary Motor Area (L)	4.57	-9, -10, 78	3855
Precuneus (L)	4.33	-10, -50, 72	
Paracentral Lobule (R)	4.23	2, -28, 69	

This table depicts regions where all subjects had less predicted grey matter as a function of more CSF SOD1. The highest *t* value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest sub-maxima *t* values in those areas are indicated. Coordinates are in MNI atlas space. Brains are oriented in neurological space.

L left hemisphere, *R* right hemisphere

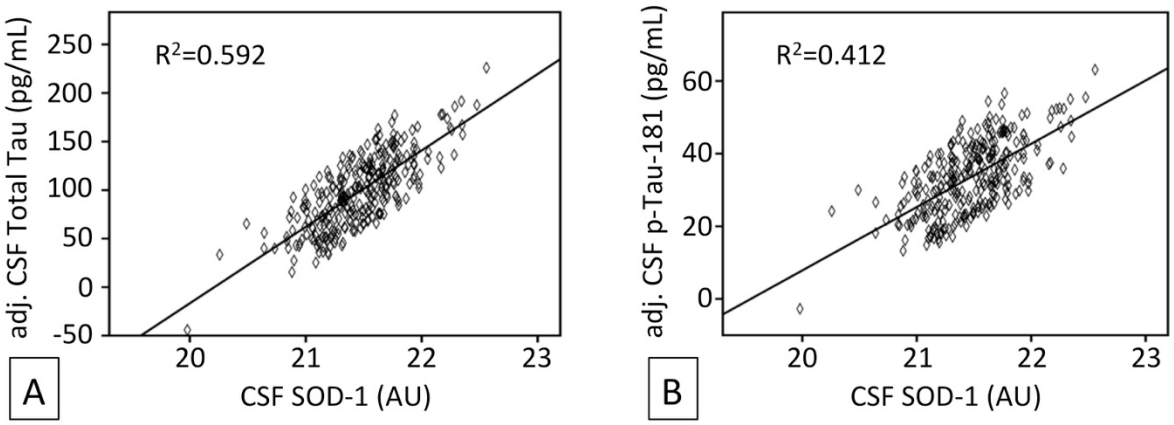

Figure 5.1: The association between higher CSF SOD1 and higher covariate-adjusted CSF total tau (A) and p-tau-181 (B) values.

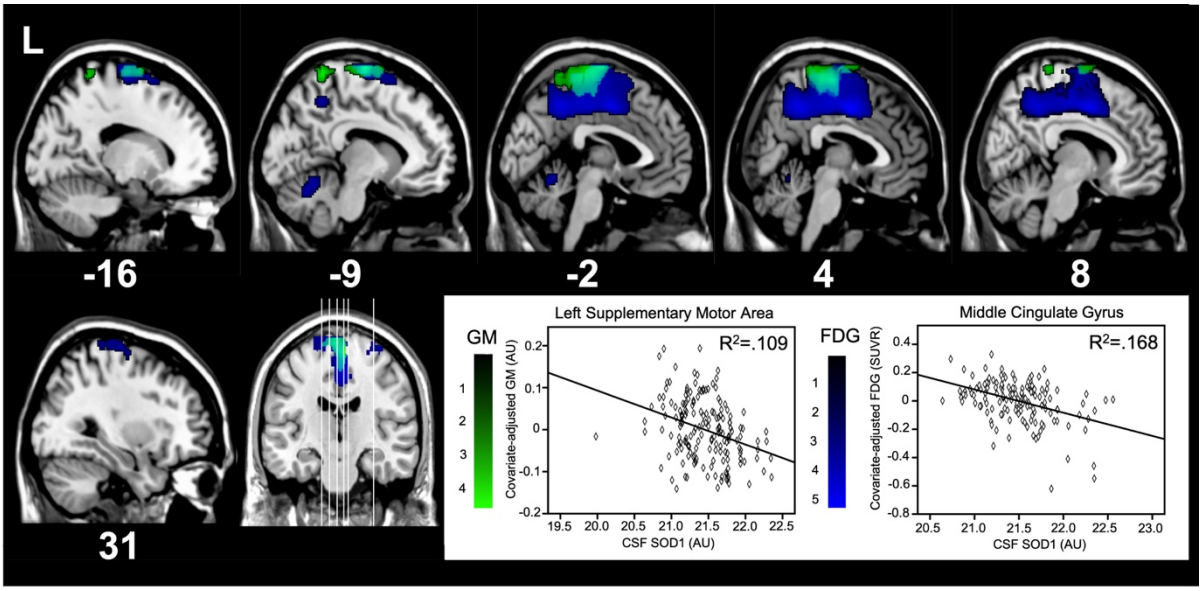

Figure 5.2: Brain areas showing less FDG metabolism ("blue") and GM ("green") as a function of more CSF SOD1. Numbers below each brain slice represent the sagittal MNI coordinates of the slice. Overlapping areas showing both less FDG metabolism and GM are teal. The GM graph depicts the relationship at the maximal voxel in the left supplementary motor area (-9, -10.5, 78) and the FDG graph depicts the relationship at the maximal voxel in middle cingulate gyrus (4, -2, 44). To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

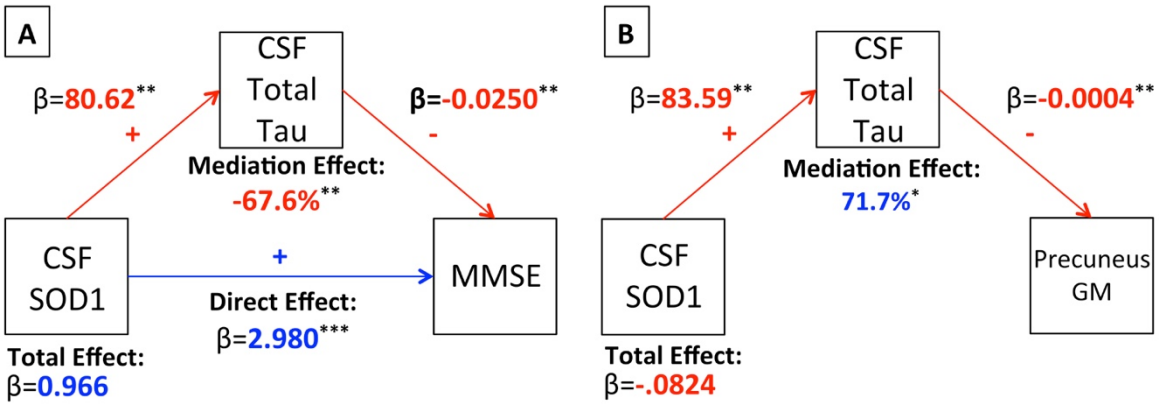

Figure 5.3: Preacher-Hayes mediation of CSF SOD-1, total tau, and MMSE scores at baseline **(A)**. Preacher-Hayes mediation of CSF SOD1, CSF total tau, and regional GM volume in left precuneus in a sub-maximum voxel (-10.5, -49.5, 72) **(B)**.

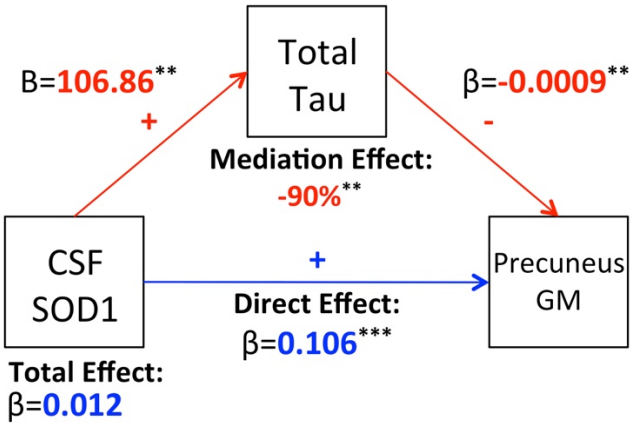

Figure 5.4: Preacher-Hayes mediation of CSF SOD1, total tau and regional GM volume in left precuneus in a sub-maximum voxel (-10.5, -49.5, 72) among participants with MCI who converted to AD within 24 months and AD participants diagnosed at baseline.

CHAPTER 6. SERUM VITAMIN B12, AND RELATED CUBILIN GENOTYPES, PREDICT NEURAL OUTCOMES ACROSS THE AD SPECTRUM

A paper in revision with the British Journal of Nutrition

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Abstract

B-vitamin supplementation has been shown to reduce brain atrophy in cognitively impaired participants. Epidemiological studies, however, show mixed findings for higher serum B12 and both cognitive and regional volume outcomes. No studies to date have

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

[†] Data used in the preparation of this article was obtained from the Australian Imaging Biomarkers and Lifestyle flagship study of ageing (AIBL) funded by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) which was made available at the ADNI database (www.loni.usc.edu/ADNI). The AIBL researchers contributed data but did not participate in analysis or writing of this report. AIBL researchers are listed at www.aibl.csiro.au.

comprehensively examined, in non-supplemented individuals, serum B12 level associations with regional neurodegeneration, hypometabolism, and cognition across the Alzheimer's disease (AD) spectrum, and if B12-related genes influence these relationships. Serum vitamin B12 was assayed from the Alzheimer's Disease Neuroimaging Initiative (ADNI) and the Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing (AIBL) in individuals who were not taking vitamin B12 or multivitamin supplements. The mean age of ADNI participants was 74.1 and 52% of participants were male; for AIBL, the mean age was 72.0 and 47% of participants were male. Voxel-wise analyses regressed vitamin B12 levels against regional gray matter (GM) volume and glucose metabolism ($p<.05$, family-wise corrected). For ADNI GM volumes, there were 39 cognitively normal (CN), 73 mild cognitive impairment (MCI), and 31 AD participants. For ADNI fluorodeoxyglucose PET (FDG-PET) images, there were 35 CN, 82 MCI and 34 AD participants. For AIBL GM volumes, there were 311 CN, 59 MCI, and 31 AD participants. Covariates were age, sex, baseline diagnosis, APOE4 status, and BMI. Single Nucleotide Polymorphisms (SNPs) in MTRR, MTHFR, TCN2, and CUBN genes were tested using PLINK and ADNI GWAS data (MAF$\geq$0.05, Alpha=.0125, corrected). In ADNI participants, higher vitamin B12 negatively predicted GM in the right precuneus, left middle and right inferior frontal gyri. In AIBL participants, higher vitamin B12 was associated with more grey matter in the right amygdala and right superior temporal pole. Higher B12 was related to less FDG metabolism in the right calcarine and precuneus. CUBN gene SNPs modified how B12 was associated with GM and FDG. Our findings provide evidence that higher B12 and related genotypes in an older population are associated with AD neuropathology.

Introduction

Deficient levels of vitamin B12 or folate lead to increased levels of homocysteine, which is a risk factor for thrombosis, microbleeds, strokes, cognitive impairment and neuronal atrophy[1; 2]. Vitamin B12, or cobalamin, normally contributes to the production of myelin in the central nervous system and fatty acid metabolism. When vitamin B12 is consumed, it first binds to haptocorrin (or transcobalamin I) to protect the B12 from stomach acid. Intrinsic factor is produced in the stomach and binds to vitamin B12 in the intestines to allow absorption into the ileum enterocytes via the membrane protein cubilin[3; 4]. Importantly, vitamin B12 is transported to the liver via transcobalamin II, where it takes place in folate/B12 dependent remethylation to facilitate the conversion of homocysteine to methionine and the subsequent methylation of DNA, proteins and lipids[5].

The literature is currently mixed about the role of vitamin B12 in brain health. On the one hand, vitamin B12 is significantly lower in both plasma[6] and CSF[7] of patients with Alzheimer's disease (AD) versus normally aging controls, suggesting that supplementation may be useful. Indeed, clinical trials have found that B12 supplementation may slow brain atrophy in Mild Cognitive Impairment, or MCI, that is considered the precursor to AD, when Omega-3 fatty acid levels are sufficiently high, perhaps by changing homocysteine levels[8; 9]. However, vitamin B12 supplementation in aged, cognitively normal adults with diabetes led to less grey matter volume in the left middle temporal pole and the left insula[10]. In addition, aged adults with mildly elevated plasma homocysteine levels showed less total brain volume after 2 years of daily supplementation with 500 μg of vitamin B12 and 400 μg of folic acid versus placebo tablet[11]. Apolipoprotein E4 (APOE4) carrier status, the

strongest genetic risk factor for developing AD, may modify the positive associations between B12 and regional GM[12].

For cognitive function, the literature is also mixed regarding B12 supplementation efficacy or its use as a biomarker to track AD-related cognitive decline. Despite controversy[13] surrounding the meta-analysis by Clarke et al.[14] in selecting rigorous clinical trials and sensitive global cognitive measures in normal aging, meta-analyses indicate that vitamin B12 supplementation may not influence cognitive decline among cognitively normal (CN) aged adults with type 2 diabetes[14], perhaps due to the mild nature of cognitive decline in normal aging and difficulty in controlling for nutritional status[15]. Vitamin B12 combined with folate does appear to have modest clinical efficacy in CN or MCI participants, however[16]. Qin and colleagues found that individuals in the top quintile for B12 intake showed increased performance in working memory, but no differences in memory or executive function tests[17]. Conversely, in a study following 765 individuals aged 85 and older over 5 years, participants who died or were unable to complete cognitive testing had significantly higher plasma vitamin B12 at baseline[18].

Wide variability in vitamin B12 associations with GM atrophy may be due to vascular and/or genetic methylation factors. Thus, it also worthwhile to see if genetic variation in vitamin B12 may explain conflicting findings for GM volume, as well as a lack of association between vitamin B12 and fluorodeoxyglucose (FDG) Positron Emission Tomography (PET)[19; 20]. We examined Single Nucleotide Polymorphisms (SNPs) among four a priori selected genes involved in B12 transport, uptake, and metabolism, CUBN, MTHFR, MTRR and TCN2[21]. We therefore systematically investigated associations between circulating B12,

regional GM and FDG metabolism, and genotypic variation related to B12-neural associations across the AD spectrum in two large cohorts spanning North America and Australia.

Methods

Participants

Data from aged adults were obtained from the ADNI database (http://adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of MCI and early AD. For up-to-date information, see http://www.adni-info.org. Written informed consent was obtained from all ADNI participants at their respective ADNI sites. The ADNI protocol was approved by site-specific institutional review boards. Data was collected by the AIBL study group. AIBL study methodology has been reported previously[22]. To eliminate the influence of supplements, especially in participants who were receiving increased care from physicians, only participants who did not report taking vitamin B12 supplements or multivitamins were included in these analyses.

Serum Biomarkers

Vitamin B12 data were downloaded at baseline. ADNI B12 levels are reported in pg/mL and AIBL levels are reported in pmol/L. All AIBL B12 values were converted to pg/mL

for consistency. Homocysteine was obtained through the ADNI and a data request from AIBL.

APOE Genotype

The ADNI Biomarker Core at the University of Pennsylvania conducted APOE ε4 genotyping. We characterized participants as having zero APOE4 alleles, one APOE4 allele, or two APOE ε4 alleles. APOE genotypic data was downloaded for AIBL participants from the AIBL.

Amyloid and Tau CSF Biomarkers

CSF sample collection, processing, and quality control of p-tau, total tau, and $A\beta_{1-42}$ are described in the ADNI1 protocol manual (http://adni.loni.usc.edu/) and Shaw et al [23]. Amyloid and tau markers were only available in a very small subset of AIBL participants, so these were not assessed.

Neuropsychological Assessment

ADNI utilizes an extensive battery of assessments to examine cognitive functioning with particular emphasis on domains relevant to AD. A full description is available at http://www.adni-info.org/Scientists/CognitiveTesting.aspx. All subjects underwent clinical and neuropsychological assessment at the time of scan acquisition. Neuropsychological assessments included: The Clinical Dementia Rating sum of boxes (CDR-sob), Mini-Mental Status Exam (MMSE), Auditory Verbal Learning Test (RAVLT), and AD Assessment Schedule - Cognition (ADAS-Cog). A composite memory score encompassing the RAVLT, ADAS-COG, MMSE, and Logical Memory assessments was also utilized[24]. Additionally, a composite executive function score comprising Category Fluency—animals, Category Fluency—

vegetables, Trails A and B, Digit span backwards, WAIS-R Digit Symbol Substitution, Number Cancellation and 5 Clock Drawing items was used[25]. These composite scores were used in formal analyses to represent memory and executive function among subjects. Out of the cognitive tests that were available for ADNI, only Logical Memory- Immediate Recall, Logical Memory- Delayed Recall, MMSE, and Global CDR scores were available for AIBL, although the same protocols were used. An executive function composite factor was available for AIBL, although it was comprised of CDR-sob, the Stroop test, the FAS test and Category Switch Total[26].

Magnetic Resonance Imaging (MRI) Acquisition and Pre-Processing

MRI scans were available for both ADNI and AIBL. T1-weighted MRI scans were acquired within 10-14 days of the screening visit following a back-to-back 3D magnetization prepared rapid gradient echo (MP-RAGE) scanning protocol described elsewhere[27]. Images were pre-processed using techniques previously described[28]. Briefly, the SPM12 "New Segmentation" tool was used to extract modulated GM and WM volume maps to Montreal Neurological Institute (MNI) space. Maps were smoothed with an 8mm Gaussian kernel and then used for voxel-wise analyses.

FDG-PET

FDG-PET images were available only for ADNI. FDG-PET acquisition and pre-processing details have been described previously[27]. Briefly, 185 MBq of [18-153-F]-FDG was injected intravenously. After 30 minutes, six 5-minute frames were acquired. Frames of each baseline image series were co-registered to the first frame and combined into dynamic image sets. Each set was averaged, reoriented to a standard 160 x 160 x 96 voxel spatial

matrix of resliced 1.5 mm^3 voxels, normalized for intensity, and smoothed with an 8mm FWHM kernel. In order to derive the standardized uptake value ratio (SUVR), pixel intensity was normalized according to the pons since it demonstrates preserved glucose metabolism in AD[29]. Normalization to the pons removed inter-individual tracer metabolism variability. The MNI template space was used to spatially normalize images using SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/).

Genomic Data Processing and Quality Control

Genomic data was only available from ADNI. Quality control of this data was conducted by analyzing Hardy-Weinberg equilibrium (HWE) accepted data for Mendelian inheritance errors. From the entire dataset, SNPs were selected for further analyses based on HWE P-value > 0.00001, MAF >0.05%, call rate 95%. Samples with greater than 5% missingness were removed. Sample genotypes were imputed using 1000Genomes data with Shapeit/Impute2 software following the protocol described previously[30]. SNPs with call rates <95 % or $R^2 \leq 0.3$ were also excluded, leaving 2,976,223 imputed and genotyped SNPs after quality control.

Statistical Analysis

For voxel-wise analysis, 2nd-level linear mixed models in SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/) tested main effects of vitamin B12 on regional GM volume and FDG, controlling for age, sex, BMI, baseline diagnosis, APOE4 status. Follow-up voxel-wise analyses were conducted on each diagnostic group, separately. The thresholds were set at $p < .005$ (uncorrected) and $P < .05$ (corrected) for voxels and clusters respectively. Results were considered significant at the cluster level. As described

previously[31], in order to reduce type 1 error, we utilized a GM threshold of 0.2 to ensure that voxels with <20% likelihood of being GM were not analyzed. For GM, Monte Carlo simulations in ClusterSim (http://afni.nimh.nih.gov/afni/doc/manual/3dClustSim) were used to estimate that 462 contiguous voxels were needed for such a cluster to occur at $p < 0.05$. For FDG voxel-wise analyses, Monte Carlo simulations in ClusterSim were used to estimate that 224 contiguous voxels were needed for such a cluster to occur at $p < 0.05$.

All genetic association analyses were conducted using PLINK v1.9 (http:// www.cog-genomics.org/plink2). The following genes were analyzed through linear associations in Caucasian participants with a phenotype of the predicted GM and FDG uptake in maximal voxels from voxel-wise analyses: CUBN, MTHFR, MTRR and TCN2. Covariates for PLINK analyses included sex, clinical diagnosis, intracranial volume, and APOE4 status. A Holm-Bonferroni threshold for significance was set of .05/4, $p < 0.0125$[32].

Non-voxel linear mixed regression was conducted using SPSS 25 (IBM Corp., Armonk, NY) to test the main effects and interactions with diagnosis and APOE4 status of vitamin B12 on cognitive scores and biomarkers. Binomial logistic regression was also used to assess the odds ratio of a given participant being diagnosed as MCI or AD versus the CN reference group. Covariates included age, sex, BMI, baseline diagnosis and APOE4 status. Years of education was also added as a covariate in models with cognitive scores.

Results

Demographics and Data Summary

Clinical and demographic data for subjects are presented in **Table 6.1a**, for both ADNI and AIBL participants. Vitamin B12 was converted to pg/mL. A sub-sample of participants had FDG data, where clinical and demographic data are listed in **Table 6.1b**.

Regional Grey Matter Volume

Voxel-wise analysis was used to regress plasma vitamin B12 against regional GM at baseline for 144 participants from ADNI and 401 participants from AIBL, who had structural GM MRI scans as well as demographic and biological data. For ADNI participants, higher plasma vitamin B12 was correlated with less GM in three significant clusters. The most significant cluster consisted of 507 voxels which was primarily in the right precuneus (**Figure 6.1)** and right posterior cingulate gyrus. The other clusters spanned the left middle frontal gyrus and right inferior frontal gyrus. See **Table 6.2** for a full listing of significant clusters. When examining each diagnostic group separately, only the MCI participants showed significant associations for B12 and GM. Higher B12 was associated with less GM in the right thalamus (k=1023). For AIBL participants, higher plasma vitamin B12 was correlated with more grey matter (k=559) in the right amygdala and right superior temporal pole. See **Table 6.3** for a full listing of significant clusters. When examining each diagnostic group separately, only the cognitively normal participants showed significant associations, with more B12 associated with more grey matter in three significant clusters, one in the right superior frontal gyrus (k=1186), one in the right precuneus (k=2105), and one in the right supplementary motor area (k=496).

Regional FDG Metabolism

FDG data was only available for ADNI participants. Voxel-wise analysis was similarly used to regress plasma B12 concentrations against FDG glucose uptake in 151 ADNI participants. Higher plasma vitamin B12 was correlated with less FDG glucose uptake in the right calcarine and right precuneus, where **Figure 6.2** illustrates the relationship at the maximum voxel in the right calcarine (24, -48, 6) (**Figure 6.2)**. See **Table 6.4** for a full listing of significant clusters. When splitting the analyses by baseline diagnosis group, no clusters survived correction for CN and AD participants. For MCI participants, there were both negative and positive associations with vitamin B12. Higher vitamin B12 was associated with less grey matter in one large cluster (k=2166) spanning the right lingual gyrus and the right thalamus. In contrast, higher vitamin B12 was associated with more grey matter in four smaller clusters, spanning the left inferior frontal gyrus (k=780), right crus 1 of the cerebellum (k=841 and 275), and the right inferior frontal gyrus (k=615).

Genotype Analyses for Vitamin B12 and Predicted Differences in GM and FDG

SNPs for genes associated with vitamin B12 uptake, transport, and metabolism were next used as predictors of interest, to see if genotypes might explain the wide variance seen in B12 associations. Linear regression in PLINK tested the additive genetic model of each SNP for associations with GM and FDG predicted values, from the voxel-wise analyses reported above. Nine SNPs in the CUBN gene were significantly associated with GM and passed Holm-Bonferroni correction, seven which were detrimental for individuals who had the minor allele and two which were beneficial (**Table 6.5**). One SNP, rs7918972 in the CUBN gene was significantly associated with FDG and passed Holm-Bonferroni correction,

which was associated with less FDG uptake (β=-0.094, p=0.0065)**.** MTHFR, MTRR, and TCN2 SNPs were not significantly associated with GM or FDG B12 predicted values.

Cognition and Biomarkers

Vitamin B12 was not significantly correlated with CD Global scores, CDR-SOB 11, MMSE, composite executive factors, or the composite memory factor for ADNI or AIBL participants. There was a significant Baseline Diagnosis * B12 interaction for predicting the RAVLT learning score for ADNI participants (F=3.080, p=0.048). Individually, higher B12 was associated with better scores in AD (β =0.0027±0.001, p=.042) but trending worse scores in MCI (β =-0.0022±0.001, p=.061).

For biomarkers, higher vitamin B12 was associated with lower plasma homocysteine levels in ADNI participants (β±SE=-.0034±0.001, p=0.001) and AIBL participants (β±SE=-0.0021±0.001, p<.001). There were no significant associations between vitamin B12 and CSF total tau, p-tau-181 or $A\beta_{1-42}$ for ADNI participants. Only a very small subset of AIBL participants had available CSF data, so these associations were not assessed.

Baseline Diagnosis: Differences in Vitamin B12 Levels

Binary and multinomial logistic regression were used to examine if plasma vitamin B12 levels predicted an increased likelihood of being MCI or AD. The reference group was CN. Serum vitamin B12 levels did not predict a higher Odds Ratio for being MCI or AD.

Discussion

We hypothesized that serum vitamin B12 levels may be a useful biomarker for AD-related brain atrophy, hypometabolism, and cognitive decline. We originally predicted that there would be an inverse correlation between higher serum vitamin B12 levels and lower

markers of AD, due to previous work from other groups[33; 34]. This hypothesis was true for AIBL participants, where higher vitamin B12 was related to more grey matter in the right amygdala and right superior temporal pole. Strikingly, in ADNI participants, higher serum vitamin B12 was instead related to less grey matter volume in the right precuneus, left middle and right inferior frontal gyri and less FDG metabolism in areas spanning the right calcarine and precuneus, areas which are adversely affected in AD. A possibility for these negative findings is that vitamin B12 is not necessarily a negative modifier of GM or FDG uptake. Instead, raised levels above an individuals' historical average may indicate ongoing systemic or neurological damage. Mosconi and colleagues did not find any correlation between vitamin B12 intake and FDG associations, although plasma vitamin B12 levels have been shown to be correlated with increased all-cause mortality in women aged 85 or greater[35]. Also, individuals with hyperlipidemia and non-insulin-dependent diabetes had significantly higher levels of vitamin B12 compared to healthy controls[36]. Additionally, higher serum vitamin B12 may be a sign of its decreased cellular uptake. Vascular damage is a common feature of AD[37], and the vascular endothelium via the CD320 receptor may mediate the homeostasis between the serum and tissue homeostasis of vitamin B12[38]. Finally, disease status may also modify uptake and metabolism dynamics of B12. For example, higher serum B12 was related to better memory performance in CN, but worse in MCI. This may be why we saw the positive relationship with GM in AIBL participants, because the distribution was strongly skewed towards CN participants. It is unclear what mechanisms might underlie these differences in either B12 transport, utilization, or metabolism.

Supplementation with B12 in individuals with dementia has been inconclusive. There was no significant improvement in memory or cognition in individuals with dementia who had low levels of vitamin B12 and were subsequently supplemented, however the supplemented individuals showed better verbal fluency scores compared to non-supplemented controls [(39)]. However, the VITACOG trial showed that B-vitamin supplementation was related to positive cognitive outcomes in individuals in the top tertile of baseline omega-3 fatty acids, compared to individuals with lower baseline omega-3 fatty acid levels, perhaps due to a synergistic effect on phospholipid production for the brain[(40)].

It has also been shown that vegetarians, though unlikely to be classified as clinically deficient, are more likely to have low-normal levels of vitamin B12[(41)]. This could likely be extended to individuals who consume plants as a larger portion of their meals, compared to meat. Perhaps vitamin B12 status may act as an indicator of the ratio of meat intake to plant intake in the diet, and this may manifest in predicting AD outcomes, which have also been linked to plant-based dietary habits[(42)]. The high intake of methionine in meat-heavy diets may provide an additional explanation. In a group of APOE deficient mice, mice that were fed a high methionine diet with high levels of B-vitamins showed significantly greater aortic plaques compared to mice that were fed a B-vitamin deficient diet, even though the first group had normal levels of homocysteine and the second group had high levels[(43)]. It is still puzzling that we still see an inverse correlation between serum vitamin B12 and homocysteine levels, even though higher B12 is correlated with worse neural outcomes. Potentially, vitamin B12 still is taken up by the liver in individuals with higher serum vitamin

B12, but their high B12 levels may be indicative of other tissues, such as the brain, being unable to transport B12 or utilize it properly.

Alternatively, genetic polymorphisms in B vitamin uptake, transport, and metabolism may modify how B12 is utilized, and affect B12 levels themselves. We found that minor allele polymorphisms in the CUBN gene tracked the association between high B12 and less regional GM. Cubulin is also an apolipoprotein receptor, and is involved in the absorption of high density lipoproteins in the kidney[44]. Perhaps cubulin polymorphisms may lead to decreased apolipoprotein reuptake in the kidneys, which may put a patient at risk for dementia.

There are several limitations of the study. First, only vitamin B12 was measured in the participants. Ideally, holotranscobalamin and methylmalonic acid would also be measured, which would indicate the level of vitamin B12 available for cellular uptake, and the absence of vitamin B12 from necessary methylation reactions, respectively[45]. Current practice in the healthcare field is to test serum vitamin B12 levels or holotranscobalamin first (if a patient has risk factors for vitamin B12 deficiency); a serum vitamin B12 level of less than 200 pg/mL is considered deficient [46]. Subsequently, current practice is for practitioners to test an additional metabolic indicator, either methyl malonic acid or homocysteine [47]. Second, it is difficult to determine the players involved in an individual's vitamin B12 levels. This can be impacted by proton pump inhibitors[48], level of animal product intake[49], genetic variability[21] as we too have illustrated. Perhaps the interaction between vitamin B12 and one or more of these variables may play a role in cognitive decline. We were also unable to assess associations with physical activity, circulating

vitamin B12, and neurological outcomes. Additionally, a limitation of the study is the lack of FDG scans and genetic data from AIBL. Lastly, because the overwhelming majority of ADNI participants are of Caucasian descent, we were only able to reliably test the interactions between genetic data and volumetric/function brain outcomes in those individuals. It would be worthwhile to determine if similar genes are implicated in an African-American cohort, as the incidence of AD is much higher among African-Americans compared to Caucasians[50].

Conclusion

This study is the first, to our knowledge, to show a negative correlation between serum vitamin B12 and brain volume and FDG metabolism across the AD spectrum. This result was unexpected, given previous B-vitamin supplementation trials showing less hippocampal atrophy[9]. Additionally, we have shown that these results may be influenced by genetic mutations related to vitamin B12 uptake and metabolism in the liver. Future research should focus on the rate of uptake of vitamin B12 into healthy and diseased neuronal cells and determine if therapeutic methods exist for improving vitamin B12 utilization in the AD population.

Authors' Contributions

K.E.M. conducted analyses and prepared manuscript. A.D.C. conducted genomic analyses and edited manuscript. A.A.W. conducted analyses and prepared manuscript.

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Tables and Figures

Table 6.1a Demographics for ADNI and AIBL participants with GM images

ADNI	**CN=39**	**MCI=73**	**AD=31**
Age	74.00 ± 5.17	73.16 ± 7.24	73.58 ± 6.19
Serum B12 (pg/mL)	439.67 ± 213.00	382.63 ± 149.22	420.51 ± 238.41
BMI	26.61 ± 4.04	25.42 ± 3.68	25.14 ± 3.90
Sex % Female	51.3%	41.4%	61.3%
% APOE 4 carriers* **	25.6%	61.7%	74.2%
AIBL	**CN=311**	**MCI=59**	**AD=31**
Age* **	71.17 ± 6.49	75.37 ± 7.25	73.23 ± 8.31
Serum B12 (pg/mL)	415.55 ± 163.63	429.70 ± 156.91	405.21 ± 181.82
BMI	26.29 ± 3.94	26.31 ± 4.54	25.86 ± 3.89
Sex % Female **	56.6%	35.6%	45.2%
% APOE 4 carriers* **	29.9%	47.5%	67.7%

Values are mean ± SD. *,**,***=p<.05, .01, .001. Chi-square analyses were conducted to examine differences between gender and APOE4 status. ANOVA was otherwise used.

Table 6.1b Demographics for ADNI Participants with FDG Images

ADNI	CN=35	MCI=82	AD=34
Age*	77.03 ± 5.05	73.54 ± 7.06	74.04 ± 7.35
Serum B12 (pg/mL)	407.40 ± 193.42	397.24 ± 181.08	467.71 ± 189.29
BMI*	27.27 ± 3.64	26.75 ± 4.39	74.04 ± 7.35
Sex % Female	40%	32.9%	47.1%
% APOE 4 carriers***	17.2%	62.2%	70.6%

Values are mean ± SD. *,**,***=p<.05, .01, .001. Chi-square analyses were conducted to examine differences between gender and APOE4 status. ANOVA was otherwise used.

Table 6.2 Regional associations of higher serum vitamin B12 and less grey matter volume in ADNI participants

Location	T value	X, Y, Z	Cluster size (voxels)
Precuneus (R)	4.21	10, -54, 27	507
Posterior cingulate gyrus (R)	3.17	8, -46, 22	
Precuneus (R)	2.70	14, -62, 33	
Middle frontal gyrus (R)	3.92	-24, 51, 6	754
Middle frontal gyrus (L)	3.35	-24, 24, 39	
Middle frontal gyrus (L)	3.24	-38, 48, 20	
Middle frontal gyrus (R)	3.85	26, 51, 3	2453
Inferior frontal gyrus (R)	3.75	44, 39, 27	
Inferior frontal gyrus (R)	3.62	51, 32, 8	

This table depicts regions where all subjects had less predicted gray matter volume per unit increase in vitamin B12. The highest t value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest t value in those areas is indicated.

Coordinates are in MNI atlas space. Brains are oriented in neurological space.

L left hemisphere, *R* right hemisphere

Table 6.3 Regional associations of higher serum vitamin B12 and more grey matter volume in AIBL participants

Location	T value	X, Y, Z	Cluster size (voxels)
Amygdala (R)	3.57	30, 4, -24	559
Superior temporal pole (R)	3.13	45, 14, -18	

This table depicts regions where all subjects had more predicted gray matter volume per unit increase in vitamin B12. The highest t value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest t value in those areas is indicated.

Coordinates are in MNI atlas space. Brains are oriented in neurological space.

L left hemisphere, *R* right hemisphere

Table 6.4 Regional associations of higher serum vitamin B12 and less FDG glucose uptake

Location	T value	X, Y, Z	Cluster size (voxels)
Calcarine (R)	3.90	24, -48, 6	479
Precuneus (R)	3.67	14, -40, 4	
Thalamus (R)	4.40	10, -32, 10	

This table depicts regions where all subjects had less predicted FDG glucose uptake per unit increase in vitamin B12. The highest t value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest t value in those areas is indicated.

Coordinates are in MNI atlas space. Brains are oriented in neurological space.

L left hemisphere, *R* right hemisphere

Table 6.5 Association between CUBN SNPs and predicted GM at the maximal voxel in the right precuneus

Chromosome	SNP	BP	A1	N	Beta	F Statistic	P value
10	rs10904831	16931344	T	111	-0.08162	-2.542	0.01249
10	rs2356587	16979380	C	111	0.02488	2.594	0.01085
10	rs1801234	16979661	C	111	0.02488	2.594	0.01085
10	rs7072262	17057766	T	109	-0.02607	-2.741	0.007229
10	rs4614335	17059826	A	108	-0.0262	-2.74	0.007252
10	rs12218279	17060676	A	109	-0.02607	-2.741	0.007229
10	rs7897550	17064992	A	111	-0.02482	-2.636	0.009651
10	rs11254331	17065357	A	110	-0.02465	-2.612	0.01035
10	rs17139621	17065761	C	110	-0.02465	-2.612	0.01035

Beta values represent the difference in the predicted value of grey matter with an increase from no risk allele to one risk allele or two risk alleles. BP= base pair location of SNP; A1= minor allele.

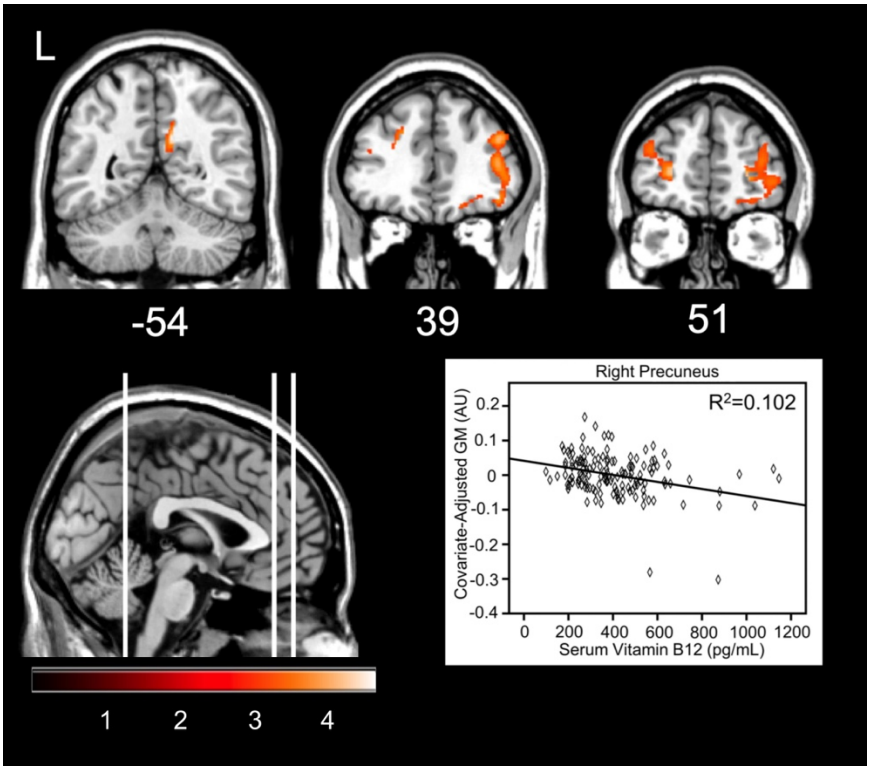

Figure 6.1 Brain areas showing less GM corresponding to increased vitamin B12. The graph depicts the relationship at a maximal voxel in the right precuneus.

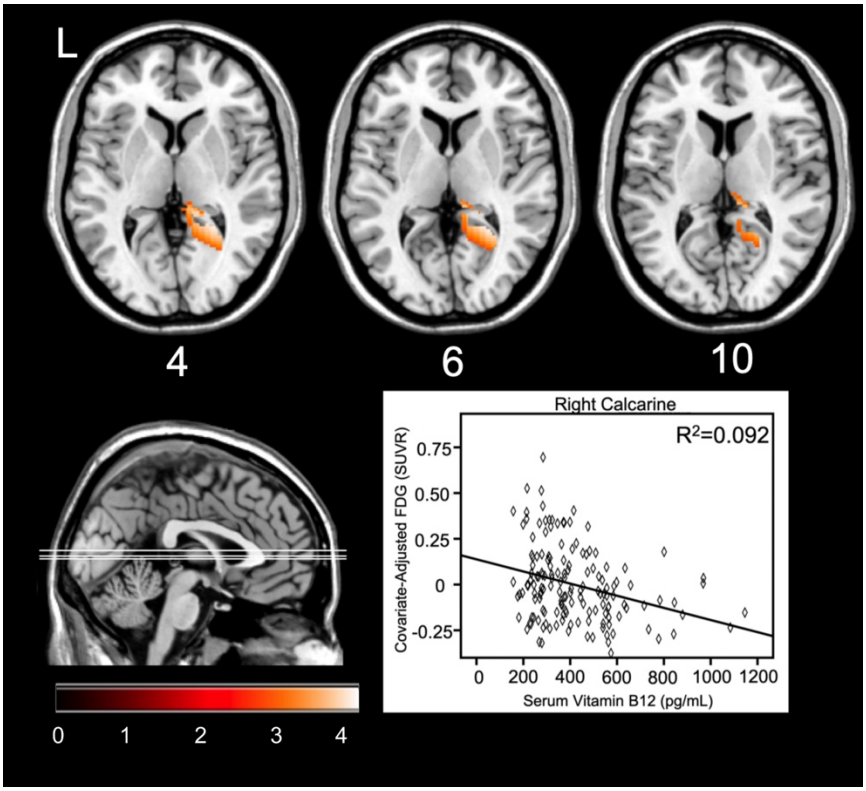

Figure 6.2 Brain areas showing less FDG metabolism corresponding to increased vitamin B12. The graph depicts the relationship at a maximal voxel in the right calcarine.

CHAPTER 7. SERUM VITAMIN B12 IS RELATED TO IMPROVED EXECUTIVE FUNCTION IN YOUNG ADULTS

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Abstract

Higher serum vitamin B12 has been shown to be related to improved cognition in cognitively unimpaired adults in middle to late age. However, relatively few studies have examined the relationship between vitamin B12 and cognition in younger adults, especially with a focus on executive function. Sixty healthy young adults were recruited across the BMI groups of lean (BMI of 18.5-24.9) and obese (BMI $\geq$30). Participants underwent a fasting blood draw and a battery of executive function related cognitive tests including the California Verbal Learning Test, EXAMINER battery, The Trail Making Test parts A and B and Wechsler Adult Intelligence- Scale Revised Digit Span test. A MANCOVA encompassing all of the digit span variables showed a significant effect of vitamin B12, while follow-up linear mixed models showed that individually, vitamin B12 modulated the digit span forward score, while covarying for age and BMI. This provides evidence that vitamin B12 may be involved in the execution of higher-order tasks.

Introduction

Vitamin B12, also known as cobalamin, is a water-soluble vitamin mostly derived from animal sources [1]. A required cofactor for methionine synthase, vitamin B12 aids in the regeneration of methionine from homocysteine, which takes place mostly in the liver [2]. Additionally, vitamin B12 is necessary for red blood cell formation, DNA synthesis, and the production of myelin in the central nervous system [1]. Vitamin B12 is able to cross the blood brain barrier in a selective manner [3].

When vitamin B12 is consumed, it is released from bound proteins in the stomach's acidic environment [4]. Intrinsic factor, which is produced in the parietal cells of the stomach, bind to vitamin B12 to transport it to its absorption site in the ileum enterocytes [4]. In the plasma, vitamin B12 travels either bound to haptocorrin or transcobalamin (called holotranscobalamin) [4]. Holotranscobalamin is the biologically available form of vitamin B12 which can be readily accessed by the cells, while vitamin B12 bound to haptocorrin is only available to the liver [4]. Risk of vitamin B12 deficiency is typically seriously considered when an individual shows serum vitamin B12 (reflective of holotranscobalamin) levels below approximately 200 pg/mL, however methylmalonic acid levels above 0.4 micromol/L may be a more sensitive indicator of vitamin B12 deficiency, because this is a barometer of the functional ability of vitamin B12 [1]. Causes of vitamin B12 deficiency include: vegetarianism/veganism, gastrectomy, malabsorption disorders and genetic polymorphisms [5].

The vast majority of research in the realm of vitamin B12 and cognition has utilized an older population, and there is a lack of information regarding the impact of serum

vitamin B12 on cognition in young, healthy adults. In a supplementation study with cognitively unimpaired individuals aged 70 or older with mild vitamin B12 deficiency, 195 participants were randomized between three groups: 1000 μg vitamin B12, 1000 μg vitamin B12 and 400 μg folic acid, or placebo for 24 weeks [6]. Individuals who were in the vitamin B12 supplement only group performed worse on memory tests at outcome visit compared to the placebo group, overall; additionally, there was no significant difference with respect to executive function or sensomotor speed between treatment groups [6]. However, in another study of cognitively normal adults, participants with low levels of vitamin B12 showed poorer scores on the block design test, an examination of spatial visualization, and letter fluency test [7]. In a cohort of individuals with HIV (mean age 51), low levels of vitamin B12 were associated with decreased scores on the Trail Making Part B test and WAIS digit span test for executive function as well as worse scores on the Peg Board Test for psychomotor processing [8]. Additionally, in a cohort of cognitively unimpaired older adults, serum methylmalonic acid was related to worse global cognitive scores, worse episodic memory and worse perceptual speed [9]. In a population of healthy individuals aged 60 or older, participants in the bottom 5% of serum vitamin B12 levels scored significantly worse on the Halstead-Reitan test for abstract thinking as well as on the Weschler Memory Test, however this relationship was not seen when assessing vitamin B12 through weighed 3-day food diaries [10]. Lastly, the genetic polymorphism apolipoprotein ε4 (APOE ε4) may modulate the effect of vitamin B12 on memory, where vitamin B12 is beneficial in non-carriers but not in carriers [11]. The purpose of this study was to determine if differences

exist in the younger adult population with respect to serum vitamin B12 and performance on cognitive tests.

Methods

Participants

Participants were a part of the Obesity, Signal Imaging, and Reactions at Iowa State (OSIRIS) study with a primary object of determining the effect of adiposity and related biomarkers on brain function using electroencephalography and cognitive tests. A secondary outcome for this study was to determine the impact of vitamin B12 on performance in cognitive tests. Subjects were recruited who met the following criteria: 18-40 years of age; lean (BMI of 18.5-24.9) or obese (BMI of 30 or above); no history of cardiovascular disease, hypertension, or diabetes; not currently taking weight reduction medication; non-smoking; and no history of major psychiatric disorders. The study ran between June of 2015 and December of 2018. All participants gave written informed consent and all study procedures were approved by the Institutional Review Board at Iowa State University.

Clinical and Cognitive Procedures

Participants underwent a fasting blood draw and samples were sent to Quest Diagnostics for assays. Anthropometrics were obtained as well as subject demographics. A light breakfast was provided and then participants took part in cognitive testing with trained research assistants. Cognitive tests included: the California Verbal Learning Test (CVLT), the EXAMINER battery, the Trail Making Test (parts A and B), the Paired Associates Test, and the Wechsler Adult Intelligence- Scale Revised Digit Span test.

Statistical Analyses

All statistical analyses were performed on SPSS 25 (IBM Corp., Armonk, NY). Covariates included: age, sex and BMI. To robustly contain type 1 error, a multivariate omnibus was initially conducted on the following categories of cognitive tests: 1. Working Memory: Wechsler digit span forward, Wechsler digit span backward, dot counting, 1-back and 2-back; and 2. Memory: CVLT learning (trial 5 minus trial 1), CVLT short-delay, CVLT long-delay and the Paired Associates Test. This allows us to determine if there is an overall significant association between all cognitive tests, allowing subsequent analyses to investigate each individual test without Bonferroni or similar corrections [(12)]. Follow-up analyses were linear mixed models regressing serum vitamin B12 levels against cognitive test outcomes.

Results

Demographics and Data Summary

Summary information is listed in **Table 7.1.** There were a total of 34 lean participants and 26 participants with obesity. There were no significant differences between groups based on age, sex, or ethnicity (% White). However, serum vitamin B12 levels were significantly higher in the lean group compared to the group with obesity.

Cognitive Test Results

The multivariate analysis with working memory variables (digit span forward, digit span backwards, dot counting, 1-back and 2-back) showed a significant effect of vitamin B12 ($F=2.76$, $p=0.030$), the effect was positive for each variable that was significant within the

multivariate analysis. The multivariate analysis with memory variables (CVLT learning, CVLT short-delay, CVLT long-delay and the Paired Associates Test) was non-significant.

For follow-up linear mixed models, there was a significant positive association between vitamin B12 and the Weschler digit span forward score (β±SE=0.003 ± 0.001, p=0.004) (**Figure 7.1**). Linear mixed models for vitamin B12 predicting digit span backwards, dot counting, 1-back and 2-back were non-significant.

Discussion

In this study, we found that vitamin B12 had a significant overall effect on working memory variables. In addition, vitamin B12 was significantly associated with the individual digit span forward score. These findings are consistent with results from Qin and colleagues, who showed that individuals in the top quintile of vitamin B12 intake had significantly better scores on the digit symbol substitution test (also tests working memory) [(13)].

A potential mechanism could be through increased cerebral blood flow with higher levels of B12. Chambers and colleagues showed that in participants with heart disease, participants who were given a 5 mg folic acid and 1 mg vitamin B12 supplement per day for 8 weeks showed increased brachial artery flow dilation, compared to placebo [(14)]. Although our participants did not have heart disease, a similar mechanism could be taking place.

Another potential mechanism could be related to the changes in white matter structure of young adults, as measured by fractional anisotropy, that Gupta and others noted in participants that were vitamin B12 deficient [(15)]. Similar results were shown in the hippocampus in another study of individuals with vitamin B12 deficiency [(16)]. Although none

of our participants were clinically deficient in vitamin B12, perhaps this could play a role to a lesser extent in participants at the low-end of normal vitamin B12 levels.

We also found that serum vitamin B12 levels were significantly lower in participants with obesity, compared to lean participants. The difference in vitamin B12 levels between lean and obese participants has been shown in previous studies. The authors postulated that the decreased vitamin B12 levels in individuals with obesity could be due to higher incidences of GERD, small intestinal bacteria overgrowth, and inflammation, which could decrease vitamin B12 absorption [17]. However, further studies are needed to confirm causation.

Several limitations of the study should be noted. First, due to budgetary constraints, we did not analyze other markers of vitamin B12 status such as homocysteine and methylmalonic acid, which would give us further insight into vitamin B12's functional capacity in an individual. Further, we did not track the dietary intake of the participants, which would have given information regarding vitamin B12 intake.

Conclusion

This is the first study to our knowledge to assess the association between serum vitamin B12 and cognitive factors in a young, non-vitamin B12 deficient, healthy population. Our findings that vitamin B12 levels are associated with improved executive function warrant future research into a potential benefit of having higher vitamin B12 status within the normal range.

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Tables and Figures

Table 7.1. Subject Demographics

	Lean Participants (n=34)	**Participants with Obesity (n=26)**
Age	24.06 ± 4.62	26.23 ± 5.50
Sex (% Female)	50%	61.5%
BMI (kg/m^2)* **	22.05 ± 1.96	34.90 ± 6.09
Ethnicity (% White)	58.8%	80.8%
Serum Vitamin B12 (pg/mL)*	689.62 ± 315.92	516.16 ± 224.41

Values are mean ± SD. *,**,***=p<.05, .01, .001. Chi-square analyses were conducted to examine differences between sex and ethnicity. ANOVA was otherwise used.

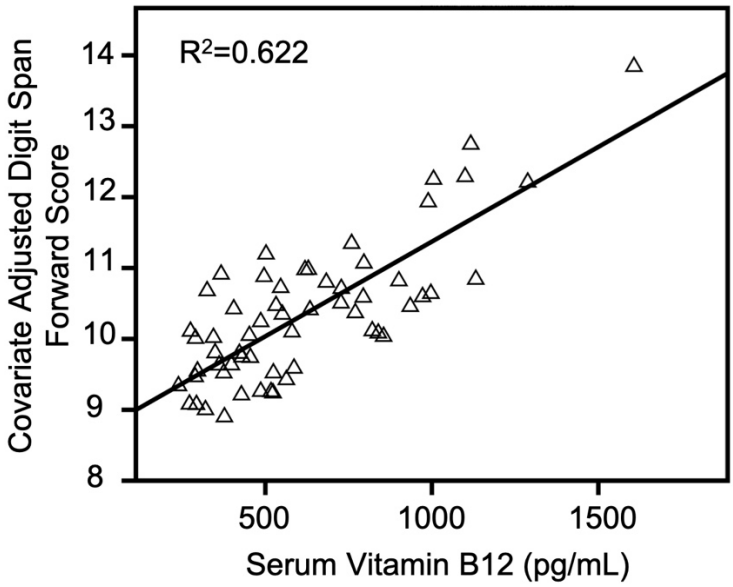

Figure 7.1 The association between serum vitamin B12 and covariate adjusted digit span forward scores.

CHAPTER 8. GENERAL CONCLUSIONS

Cognitive decline and AD have been repeatedly linked to diseases such as type 2 diabetes, hypertension, dyslipidemia, and metabolic syndrome [1; 2; 3]. Metabolic dysfunction is also correlated with increased oxidative stress and inflammation, both in the brain and throughout the body, which is correlated with the accumulation of amyloid plaques and tau tangles [1]. The studies reported in this dissertation add evidence to the current body of knowledge that the markers of metabolic dysfunction (autotaxin and insulin-like growth factor binding protein 2) correspond with AD outcomes and further emphasize the importance of nutrition-related interventions that may improve metabolic function and lessen risk of AD or progression of the disease. Oxidative stress on its own is strongly implicated in the structural and functional changes that are seen in the brain in AD. Our report of the impact of superoxide dismutase 1 on AD outcomes provide corroboration to an overwhelmed antioxidant system in AD that cannot keep up with growing amounts of reactive oxygen species. Another avenue that interventions should investigate is dietary approaches to strengthen the body's antioxidant system, such as increased intake of berries and leafy greens, and reduce its workload via lower levels of oxidative stress.

In addition, high levels of serum homocysteine have been associated with both cardiovascular disease and AD in many studies [4; 5]. Mechanisms that have been suggested include: excessive excitation of neurons and neuronal death, oxidative stress, damage to the vasculature, DNA damage, and the initiation of formation of amyloid and tau proteins [6]. Because vitamin B12 is highly involved in maintaining low homocysteine levels, it has been hypothesized that vitamin B12 may be protective of AD [7]. However, our findings

related to vitamin B12 and neurologic outcomes are conflicting. While serum vitamin B12 levels were related to improved cognition in younger adults, the relationship was largely deleterious in older adults across the AD spectrum. While it is our suspicion that vitamin B12 levels may be reflecting systemic issues or decreased cellular uptake and not causative of AD pathology, clearly more research is needed in this area and vitamin B12 supplements should be cautioned in the older population that is not deficient.

Although a pessimistic view of AD research outcomes is commonly held due to modest improvements in current practice and pharmacologic treatments available, there is promise for nutritional modifications in slowing or halting disease progression [8; 9]. In addition, each biomarker that is identified can aid in accurately noting preclinical changes so patients have the opportunity to make dietary and lifestyle changes, such as increasing physical activity to decrease IR, that may change their neurological trajectory. Multidisciplinary teams that include physicians, registered dietitians, physician assistants and nurse practitioners, nurses, socials workers, and other important health care professionals are crucial in these interventions and these groups are the leaders of progress in AD research.

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APPENDIX. INSTITUTIONAL REVIEW BOARD APPROVAL: OBESITY, STRUCTURAL IMAGING, AND REACTIONS AT IOWA STATE (OSIRIS) STUDY

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Institutional Review Board
Office for Responsible Research
Vice President for Research
1138 Pearson Hall
Ames, Iowa 50011-2207
515 294-4566
FAX 515 294-4267

Date: 6/26/2015

To: Dr. Auriel Willette
224 Mackay Hall

From: Office for Responsible Research

Title: Obesity, Structural Imaging, and Reactions at Iowa State (OSIRIS) study

IRB ID: 15-101

Approval Date:	6/26/2015	**Date for Continuing Review:**	6/15/2016
Submission Type:	New	**Review Type:**	Full Committee

The project referenced above has received approval from the Institutional Review Board (IRB) at Iowa State University according to the dates shown above. Please refer to the IRB ID number shown above in all correspondence regarding this study.

To ensure compliance with federal regulations (45 CFR 46 & 21 CFR 56), please be sure to:

- **Use only the approved study materials** in your research, **including the recruitment materials and informed consent documents that have the IRB approval stamp.**
- **Retain signed informed consent documents for 3 years after the close of the study**, when documented consent is required.
- **Obtain IRB approval prior to implementing any changes** to the study by submitting a Modification Form for Non-Exempt Research or Amendment for Personnel Changes form, as necessary.
- **Immediately inform the IRB of** (1) **all serious and/or unexpected adverse experiences** involving risks to subjects or others; and (2) **any other unanticipated problems involving risks** to subjects or others.
- **Stop all research activity if IRB approval lapses**, unless continuation is necessary to prevent harm to research participants. Research activity can resume once IRB approval is reestablished.
- **Complete a new continuing review form** at least three to four weeks prior to the **date for continuing review** as noted above to provide sufficient time for the IRB to review and approve continuation of the study. We will send a courtesy reminder as this date approaches.

Please be aware that IRB approval means that you have met the requirements of federal regulations and ISU policies governing human subjects research. **Approval from other entities may also be needed.** For example, access to data from private records (e.g. student, medical, or employment records, etc.) that are protected by FERPA, HIPAA, or other confidentiality policies requires permission from the holders of those records. Similarly, for research conducted in institutions other than ISU (e.g., schools, other colleges or universities, medical facilities, companies, etc.), investigators must obtain permission from the institution(s) as required by their policies. **IRB approval in no way implies or guarantees that permission from these other entities will be granted.**

Upon completion of the project, please submit a Project Closure Form to the Office for Responsible Research, 1138 Pearson Hall, to officially close the project.

Please don't hesitate to contact us if you have questions or concerns at 515-294-4566 or IRB@iastate.edu.