Associations between metabolic risk factors and indicators for Alzheimer's Disease pathology in a middle aged cognitively health cohort

BY

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Abstract

Dementia due to Alzheimer's disease (AD) is a devastating condition characterized clinically by progressive cognitive impairment and pathologically by amyloid plaques, neurofibrillary tangles and severe atrophy. While AD is the leading cause of dementia worldwide, only 5% can be attributed to a genetic cause. Causes of the other 95% of AD cases remains incompletely understood. Along with a lack of antemortem definitive diagnostic techniques, there are no effective long-term treatments available. Age is the number one risk factor for AD, and approximately 50% of AD patients are known to harbor the e4 allele of the APOE gene; however, once the disease has manifested clinically there are limited options for successful long-term interventions.

Diabetes mellitus (DM), obesity, and associated conditions such as insulin resistance when present in middle age, are associated with a greater risk of dementia and brain changes that differ from healthy aging. However, the question remains: how do these midlife factors affect neuropathological burden in middle age? Given the potential of modifying these risk factors at midlife, my research explored the pathophysiological mechanisms involved in the link between insulin resistance and obesity at middle-age and AD pathology using molecular and imaging biomarkers. Specifically, my studies were aimed at investigating how metabolic risk factors may affect the accumulation of amyloid and neurofibrillary tangles, alter brain structure and blood flow, and influence cognition in middle age. Data were collected from participants in the Wisconsin Registry for Alzheimer's Prevention (WRAP) study. My

studies included a number of techniques and measures, including magnetic resonance imaging (MRI), proteins assayed from cerebral spinal fluid (CSF), neuropsychological testing, as well as proteins assayed in blood and medical history data obtained from participants by trained physicians, scientists, psychologists and medical professionals to assure the highest quality data.

The first study was conducted to assess the effects of insulin resistance on cognitive decline over a 4-10 year period, and demonstrated that indeed, insulin resistance is associated with cognitive trajectories. The next three studies were aimed at identifying changes in AD biomarkers in CSF and brain imaging as predicted by insulin resistance, body mass index and abdominal obesity. The main findings indicate that elevated insulin resistance at midlife may affect amyloid processing as indicated by altered CSF levels, as well as acting via a tau-related mechanism, an effect that differed by APOE e4 genotype. Given that tau in CSF is likely originating from axons, this finding was followed up by a neuroimaging study employing diffusion tensor imaging to examine the effects of insulin resistance and APOE e4 on neural microstructure. Similar to the findings in CSF, the effects of insulin resistance on microstructure differed by APOE e4 genotype. Finally, my studies of abdominal obesity suggest that this risk factor is not associated with amyloid or tau pathology as measured in CSF, nor microstructural changes, yet central obesity was associated with moderate effects on cerebral blood flow. In conclusion, the work presented in my thesis demonstrates a robust effect of insulin resistance on cognitive and biomarker outcomes in midlife, which may explain

the increased risk of developing AD. This work also provides new data that support the development of intervention trials aimed at modifying risk factors in midlife, to potentially delay or prevent AD.

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"Place your hand over your heart, can you feel it? That is called purpose. You're alive for a reason so don't ever give up." – Unknown

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Chapter 1: Background

Alzheimer's Disease

Dementia due to AD is a distressing neurodegenerative disorder, commonly presenting clinically with severe and progressive memory loss, decreased executive function and eventually culminating in the loss of control of all bodily functions. An estimated 5.4 million Americans suffer from AD, and that number is growing at an estimated rate of one case every 66 seconds [1]. AD is also costly, with a total estimated cost of $236 billion dollars spent on care, assessment and therapy, in 2016 [1]. Surprisingly, dementia due to AD is the only top ten leading cause of death with no known prevention, cause, or cure. Now, the scientific community is on a hunt to identify underlying mechanisms of AD pathophysiology aiming to decrease incidence of AD as the American population continues to age.

Causation of most forms of AD is unclear. Insights from less common forms of AD have aided in directing science to distinct cellular mechanisms. There are a small proportion of AD cases (1-2%) that have a known genetic point mutation causing early-onset AD (EOAD)[2]. EOAD may present in patients as young as 30 although cases remain relatively rare. Autosomal dominant forms of AD include mutations in amyloid precursor protein (APP) and presenilin genes. Chromosomal abnormalities such as trisomy 21 are also associated with EOAD[3]. While EOAD is not the predominant type of AD, its presence has facilitated some of the mechanistic knowledge of AD. EOAD cases primarily point toward amyloid as a primary pathological mechanism, given that the genes implicated in EOAD are all related to APP or APP processing. The amyloid

hypothesis encompasses this perspective and purports that the cascade of neurodegeneration observed in AD is due in large part to the accumulation of amyloid plaques. Although there are striking associations between amyloid and EOAD, the same relationships are less clear for the more common form of AD cases with a much later age of onset, late onset AD (LOAD). The cause of LOAD is multifactorial leaving scientist with clues and hints about the underlying etiology of AD.

Risk factors provide valuable evidence for assessing the trajectory of risk for various individuals. The average age of onset for LOAD is about 75 years and is considered a disease of aging. Indeed, age is the top risk factor for LOAD with other risk factors including, maternal family history, sex, and education[4, 5]. Another important risk factor is the ε4 isoform of the *ApolipoproteinE* (*APOE ε4*) gene located on chromosome 19, as approximately 50-60% of AD cases involve the APOE4 gene[6]. The protein product of this gene is Apolipoprotein E (ApoE) a lipoprotein present on chylomicrons and intermediate density lipoproteins, both responsible for transportation of blood lipids specifically cholesterol[7]. Molecular and biochemical understandings of how these risk factors lead to AD are under investigation in a plethora of scientific models. Picking apart each risk factors contribution to the development of dementia will open more doors for preventions and cures. Despite accumulating years of research concerning AD[1], it remains difficult to predict who will develop dementia due to AD and who will age normally. In addition to age, risk factors include family history, risk

[1] Late onset AD is also known as sporadic AD, for the remainder of the paper, I will refer to LOAD or sporadic AD simply as AD as this work did not consider cases of EOAD.

genes, and metabolic disturbances like diabetes and obesity while other cases of AD are associated with no distinct known risk profile. With our data, we aim to help distinguish and identify risk factors at midlife at may enhance the likelihood of developing dementia due to Alzheimer's disease. While many of the pathophysiological details remain elusive, decades of investigation have been dedicated to identifying and characterizing the histopathology of AD.

Currently, a diagnosis of dementia AD can only be confirmed at autopsy by the presence of the neuropathological hallmarks of AD. In 1906, German psychiatrist Alois Alzheimer characterized neuropathology in the brain parenchyma of a patient he first met in 1901 with severe short-term memory loss, behavioral changes and, personality shifts. Upon his autopsy, he could not help but notice some peculiar histology lurking amongst the neural forests of his patient[8, 9]. That histology became known as amyloid plaques and neurofibrillary tangles, the defining histopathological elements for dementia due to AD versus other dementia etiologies. Amyloid plaques (APs) are composed of sheets of misfolded Amyloid β (Aβ) protein. Plaques have a predilection for depositing in the cerebral cortex in the extracellular spaces. Chronologically, initial deposits are located in the basal areas of the cortex and significant hippocampal involvement does not occur until later staging of the disease[10]. In turn, NFTs are a buildup of hyperphosphorylated tau protein. Tau protein normally functions to help regulate microtubule dynamics in axons, attaching and detaching to the tubulin as the cells environment changes. Accumulation, of tau fragments, begins in the

transentorhinal cortex before spreading to the cortex and then expanding to the remainder of the temporal lobe, frontal lobe, and with advanced AD, the parietal and occipital lobes [10]. The buildup of AD neuropathology begins far before dementia symptoms become clinically relevant. A movement in the field is to identify factors at midlife that may modulate the accrual of NFTs and APs before dementia and use that information to potentially slow or prevent progression of AD.

Mechanisms that control the deposition of the NFTs and APs remain unclear. However, there is increasing evidence for insulin as an independent regulator of protein processing and degradation mechanisms. Consequences of obesity may also affect the accumulation of these pathological markers. These findings and others, lead us to inquire about the role of insulin and obesity at midlife in AD.

Diabetes, Insulin Resistance, and AD

Epidemiologic studies provide substantial evidence for a connection between AD and diabetes. Clinical evidence found that AD patients were hyperinsulinemic and hyperglycemic had lower insulin in the CSF, higher plasma insulin and disrupted energy storage[11-13].

The incidence of Diabetes Mellitus type 2 (DM2) is on the rise. According to the American Diabetes Association, 18 million people have been diagnosed with Diabetes and 79 million have pre-diabetes. DM2 accounts for 90% of diabetes diagnoses in the United States. One in three Americans has pre-diabetes, the prodromal syndrome to DM2. Diabetes develops when the body can no longer regulate the levels of blood

glucose through hormone signaling of insulin. Insulin is a peptide hormone released by the pancreas when blood sugar levels rise. Following meal digestion and absorption, glucose, lipids and proteins levels increase in the blood stream. Glucose sensing cells of the pancreas rely on glucose transporter-2 (GLUT2) channels, which facilitate diffusion of glucose from the plasma into pancreatic β-cells[14]. After amplification of the signal through secondary molecular modulators, preformed insulin is released into the blood stream. Upon arrival at the target tissue, insulin binds to the insulin receptor on muscle and adipose tissue leading to the insertion of stored GLUT4 channels[15]. Increased GLUT4 channels increase the influx of glucose into target tissue where it can be utilized or stored for later use in polymeric glycogen. Disruption of insulin receptor signaling prevents proper handling of elevated blood glucose.

DM2 develops after prolonged hyperactivation of the insulin hormone signaling process. Insulin receptors are downregulated, glucose remains elevated in the blood and eventually the pancreatic cells can no longer compensate for the excessive demand of insulin. Insulin resistance is the result of decreased responsiveness of target tissue to insulin and is an essential feature of DM2. Insulin resistance occurs when the cell insulin sensitivity changes such that increasing amounts of insulin are needed to maintain healthy levels of post-prandial glycemia. Peripheral IR causes dysregulation of glucose, which has devastating consequences to the kidneys, liver, and brain[16]. There are many complications of DM2 including heart disease and stroke, blindness, kidney disease, and nervous system disease[17]. Independent of the cardiovascular(CVD)

concern, having DM2 in midlife doubles the risk of developing AD later in life. Equally concerning is the prevalence of pre-diabetes, suggesting underlying pathophysiology takes years to manifest as fulminant DM2. We sought to analyze effects of insulin resistance in individuals without DM2 as IR may affect the progression of AD in spite of a DM2 diagnosis. Our work sought to identify associations between insulin resistance and key features of AD.

Increasing evidence suggests IR contributes to the pathogenesis of AD, though details about specific mechanistic links remain unclear. IR correlates with increased amyloid plaque deposition, up-regulation of kinases involved in tau phosphorylation as well as increases in oxidative phosphorylation, decreased synaptic activity, in addition to myelin and axonal changes[18, 19]. Concerning insulin resistance and amyloid, several studies suggest that insulin resistance is implicated in amyloid accumulation[20-23]. In transgenic mouse models, diet-induced insulin resistance promoted the generation of amyloidogenic β-amyloid peptides, Aβ1-40, and Aβ1-42 and increased amyloid plaque burden[24]. Likewise, diet-induced insulin resistance is associated with increased glycogen synthetase kinase activity (GSK-3), a regulator of γ- secretase activity, the final enzyme in Aβ generation[20]. The function of insulin signaling in Aβ production has also been tested in transgenic mouse models exhibiting normal insulin signaling showing reductions of Aβ accumulation in the hippocampus,[25] providing molecular proof of the involvement of insulin signaling in amyloid accumulation.

Insulin resistance has also been linked to decreased amyloid clearance[26]. Peaking curiosity focuses on one prominent mechanism, the action of the insulin-degrading enzyme (IDE)[27]. IDE is a metalloproteinase classically thought to be focused on insulin clearance[19]. Typically, IDE will degrade deposited Aβ and amyloid plaques clearing them from the brain parenchyma[28]. The pancreatic β-cell response to IR is to increase insulin secretion compensating for decreased insulin efficacy [29]. Since IDE has a much higher binding affinity for insulin, in the presence of hyperinsulinemia, IDE preferentially binds insulin leaving less active sites for amyloid binding[30]. Experiments in IDE knockout mice show increased cerebral accumulation of amyloid as a consequence of missing IDE [28]. IDE is one of a few enzymes, typically regulated by insulin signaling, involved in AD pathogenesis. Further investigation of separate contributions is needed to clarify the particulars, which may provide novel therapeutic targets for AD.

According to animal studies, IR is a significant factor in amyloid pathogenesis, however, when assessing these relationships in humans, relationships continue to be contemplated. In a Japanese study of postmortem brains, insulin resistance correlated with increased amyloid plaque burden in the cerebrum of AD patients compared to age-matched controls[31]. Such findings support a link between IR and amyloid, although other studies examining the relationship between T2DM and amyloid have not shown an effect, possibly due to the confounds of medication usage[32]. Studies examining exogenous insulin administration as a therapy for cognitive impairment in

AD also exhibited an effect on CSF levels of amyloid peptide [33-35]. Further, other investigators report effects of insulin levels and insulin resistance on amyloid precursor protein, the Aβ parent peptide[36, 37]. The combined animal and human studies suggest insulin has more of a role in AD pathogenesis than merely energy and metabolism regulation. Most of the studies analyzed comparisons between those who already had AD and a healthy aging cohort. Uniquely, our study aimed to investigate pre-disease during middle age, when pathology presumably begins to accumulate.

There is growing evidence supporting a role for insulin in synaptic function specifically in learning and memory [19, 38]. Further, IR is implicated in tau and NFT pathology due to aberrant downstream regulatory signaling. Insulin signaling is thought to affect tau phosphorylation through downstream signaling regulation of GSK-3 β[39]. Intact [40] insulin signaling leads to phosphorylation of GSK-3β, inactivating the kinase. Decreased phosphorylation of GSK-3β, which occurs in the setting of IR, leads to a hyperactive kinase. Tau is a substrate of GSK-3β; thus, when the kinase is not shut down, it will continue to phosphorylate the tau molecule[41]. Hyperphosphorylated tau is a principal constituent of the paired helical filament (PHF) and the NFTs. Other evidence shows insulin resistance affects the phosphorylases responsible for the dephosphorylation of tau[42]. Literature considering insulin's role in NFT generation is sparser than the research on amyloid; however, the evidence to date does suggest that IR has significant effects on the structure and function of axons and synaptic function.

In sum, animal and human studies suggest insulin signaling abnormalities and insulin resistance contribute significantly to the pathogenesis of sporadic AD. Animal models are ideal for investigating molecular mechanisms. However, it's important to note that wild-type rodents do not develop AD, or plaques and tangles, they must be genetically altered to express AD pathology. This fact makes the investigation in humans imperative for relating the natural history of the disease to the human experience. Little is known about the involvement of IR in preclinical AD, but it is believed that these processes may be in play long before severe progressive memory decline. In the next section, I will consider an associated metabolic risk factor, obesity.

Obesity and AD

There is an increased risk of developing dementia for individuals who are obese at mid-life versus normal weight individuals. This is concerning, given that obesity is an increasing epidemic in the United States. According to the World Health Organization (WHO), more than half a billion people suffer from obesity worldwide[43]. Although obesity and IR are closely related, and DM2, heart disease, and stroke risk increase with increasing body mass index (BMI), the effects of these risk factors may work through independent mechanisms.

Obesity at mid-life is associated with poorer cognitive performance in older adults [44], and accruing research has demonstrated that obesity alters microstructural and macrostructural brain anatomy. Evidence supporting a link between body weight and AD suggest that weight loss is a predictor of AD clinical symptoms (rather than weight

gain), and many AD participants are underweight. However, these effects appear closer to disease onset, and may be the result of altered brain metabolism, leading to a physiological anorexia [45, 46]. Less is known about the link between weight and AD-related changes in midlife, although studies have found increased risk of developing AD (hazards ratios from ~1.4to 3.6) [47-50] when obesity is present at midlife. The question of interest is how is this increased risk conferred and what mechanisms are involved during mid-life?

Obesity has the potential to affect brain health via several, perhaps overlapping, mechanisms. First, the vascular effects that are observed in the periphery likely extend to the brain. The effects of obesity may also be a consequence of altered adipokine and hormone profiles. Adipokines are small molecules secreted by adipocytes, fat cells, that lead to substantial changes in overall metabolism and inflammation {Balistreri, 2010 #76}. One hormone to consider more carefully is leptin, a hormone that regulates energy balance by inhibiting hunger. Leptin crosses the blood brain barrier and seems to have a protective effect for neurons {Greco, 2008 #78}. Over time, prolonged elevation of leptin can lead to leptin resistance, decreasing the protective effects and potentially playing a role in obesity and possibly AD, given that animal studies have shown links with Aβ {Fewlass, 2004 #77}. Finally, obesity may have an effect via inflammatory mechanisms. Visceral fat is of particular interest in this context, as it has been shown to elevate peripheral levels of cytokines. While much of this work has been completed in animal models, little is known about the effect of midlife obesity on brain health and

development of neuropathology of AD. Thus, the studies conducted as part of this thesis examined both IR and obesity.

Summary

As communicated in the afore-written text, AD, DM2 and obesity are all rising concerns for Americans. Connections between metabolic disturbances, cognition and AD pathology have been observed across a variety of model organisms. A majority of human research has focused on post mortem accumulations. Given the vast expanse of technology such a immunoassays of serum and CSF, neuroimaging and comprehensive neuropsychological testing, we can explore correlations within a human cohort. Further, we can assess the accumulation of antemortem AD pathology, providing a better understanding of the pre-clinical disease, giving insights into the natural progression of AD starting at midlife. Significant strides in research are necessary to fully examine the consequences of metabolic dysfunction at midlife and development of AD dementia later in life. Thus, we found it imperative to use the tools provided by the UW Alzheimer's Disease Research Center(ADRC) for investigating midlife effects of IR and obesity.

The next section will outline my specific aims in the order the studies are presented in the full document.

Specific Aims

Aim1: Determine the effect of IR and central obesity on cognitive function in middle age adults.

Research indicates that being obese or having DM2 in middle age increases the risk of later life AD-related cognitive decline. Having DM2 at middle-age doubles the risk of developing dementia due to AD later. Preliminary studies assessing insulin as a therapeutic have shown improvements in cognition of AD patients versus control. Using our most significant predictors, insulin resistance, waist circumference, and body mass index, we expect to uncover the earliest cognitive changes associated with DM2 and obesity. Our data includes comprehensive neuropsychological testing, measures of blood peptides, and medical history information. We propose to analyze data acquired from a cohort of middle-aged adults enriched for a family history of AD to determine potential contributions of midlife metabolic dysfunction to cognitive decline.

Aim2: Determine the extent to which measures of IR and obesity in middle age affect markers of Alzheimer's disease pathology, as measured by CSF biomarkers of amyloid and NFTs.

IR and obesity are proposed to interfere and enhance mechanisms involved in the accumulation of plaques and tangles. IR and obesity have the potential to cause pathological changes in animal models, but little is discussed regarding the role of metabolism and AD pathology in middle-aged cognitively healthy adults. Biomarkers of interest include amyloid precursor protein, Aβ, total tau and phosphorylated tau. With data collected from a middle-aged cohort, we plan to assess the relationships

between measures for insulin resistance, obesity and CSF biomarkers for AD pathology. The proposed studies will provide novel insights into relationships between metabolism and AD biomarkers.

Aim3: Examine the effects of IR and central obesity on brain microstructure at middle age

Diffusion Tensor imaging (DTI) studies in humans have shown white matter microstructure alterations in subjects with DM2 and obesity. DTI is an imaging method sensitive to microstructural changes in the brain before gross anatomy is affected. The proposed studies aim to examine the relationship between brain changes and metabolic risk factors at middle age.

General Methods

To investigate our questions in a human cohort, we utilized data collected from the University of Wisconsin Alzheimer's disease research center (ADRC). The ADRC has many participants enrolled in many different studies, with the primary goal being to understand the natural course of AD and factors that may accelerate, or increase the risk of developing dementia. All of the participants analyzed for this thesis were part of a cohort designed to examine mid-life relationships between subjective and objective measures and AD pathology. Scientist and physicians followed participants over many years with hopes of identifying genetic and environmental components of the AD disease process. Each participant underwent a comprehensive neuropsychological

examination, blood and cerebrospinal fluid collection and neuroimaging at each visit. The following contents of this chapter provide more detailed insight as to the rationale behind choosing these measures to answer our questions.

Participants

All of the following studies were conducted using data collected from participants in the Wisconsin Registry for Alzheimer's Prevention (WRAP) study. The WRAP is a longitudinal cohort study of participants who were middle-aged and cognitively asymptomatic at enrollment, with and without a parental family history of AD. A significant goal of the WRAP study is to use longitudinal data to create a preclinical picture AD and the factors that increase the probability that any one person will develop the disease. The WRAP study provides an exciting opportunity to identify factors that could be modified to help delay the onset or even prevent the development of the disease during one's lifetime.

Cognitive Testing

Neuropsychological tests provide confirmatory evidence of the diagnosis of dementia and contribute to assessing the course and response to therapy. In fact, neuropsychological testing is the primary determinant of probable AD. In the clinic, if dementia is suspected a physician can administer the mini mental status exam to screen for those who need further testing to determine dementia status. If cognitive impairment is suspected, a more comprehensive battery of exams can help distinguish between various dementia etiologies. The main goal of

neuropsychological testing is to determine abnormal cognitive domains, which can give insight into the course of the disease. Cognitive testing has proven to be a highly accurate method for assigning a clinical diagnosis of AD[51]. However, we are focused on the preclinical stage, which is less explored, especially in a cohort such as WRAP.

For a patient, cognitive decline is their first sign for them that brain function is decreasing abnormally. In many cases, a significant other or spouse notices the decline before a patient becomes aware of their progressing mental disabilities. Before the realization of a cognitive deficit, accumulating AD pathology may cause more insidious cognitive dysfunction invisible to the clinician as a result of the current cognitive tests. If there is a surmounting accumulation of AD pathology with no impairment, you may never see an AD patient until cognitive impairment has progressed to a severe not much can be done. Identifying disease processes earlier in the course of the illness provides hope for slowing, reversing or preventing progression into advanced AD especially if modifiable risk factors are involved. We hope to identify any relationships between metabolic risk factors and cognitive deficits in middle age providing novel insights into the main cognitive domains affected earliest by metabolic dysfunction.

Cerebrospinal Fluid

Conducting studies in humans require innovative methods for the measurement of molecular activities underlying brain function and dysfunction. Cerebral spinal fluid, (CSF) is the liquid that immerses, washes, lubricates and cushions the brain. CSF is produced by specialized endothelial cells in the choroid plexus and plays a role in removing deposits of material [52]. CSF can be assayed to examine its contents for peptides, glucose, and other small molecules. We can access CSF through a lumbar puncture, and measure the peptide and metabolite content using immunology based assays. CSF amyloid beta 1-42, total CSF tau protein and P-Tau$_{181}$, have all been investigated in the context of AD and other neurodegenerative conditions. More recent studies focus on the potential for a biomarker risk panel aimed at identifying cases of preclinical AD. These biomarkers may offer a way to profile various stages of AD and other neurologic diseases. Distinct patterns of biomarker concentration exist amongst patients with AD and mild cognitive impairment (MCI)[53], an intermediate stage between normal aging and fulminant dementia. Other studies have demonstrated moderately high specificity and sensitivity of CSF biomarkers for predicting the decline from cognitively stable to MCI, and conversion from MCI to AD. CSF is an innovative method for obtaining information regarding molecular and cellular processes occurring in the brain. The methods sections of each study provide comprehensive and detailed explanations of all the general methods as they pertain to each research question.

Neuroimaging

Advanced neuroimaging techniques have opened up new frontiers for in-vivo

assessment of brain pathology. Neuroimaging is imperative to the investigation in neurodegenerative disease, specifically in early detection, and are expected to inform diagnosis and prognosis[54, 55]. Patients with AD lack a definitive diagnosis until confirmation through the presence of NFTs and amyloid plaques at autopsy. In the meantime, a diagnosis of probable AD requires assessment of cognition, activities of daily living, anecdotes from significant others or spouses and observance of clinical progression {McKhann, 2011 #79}. While this has been the method of diagnosis for several years, it is not 100% accurate. Neuroimaging techniques are currently used clinically to rule out other causes of dementia when diagnosing AD (for example presence of a tumor or stroke)[56, 57]. However, in the research setting, neuroimaging can be used to 1) determine the extent of in vivo brain alterations and 2) be used as a biomarker of AD progression to help potentially determine AD status before significant cognitive decline[58]. Imaging biomarkers of interest include positron emission tomography, for detecting amyloid plaque and tangle load, as well as MRI for assessing hippocampal volumes, blood flow, and microstructural changes[59-62].

An imaging method of particular interest is diffusion weighted imaging, an imaging technique that is well known in the context of assessing cerebral ischemia clinically an experimentally for assessment of brain microstructure health. Diffusion tensor imaging (DTI) models the random molecular movement of water, or water diffusion[63]. Alterations in the cell membrane, cell density, dendritic branching, and myelin structure can change the relative movement of water throughout the various tissues,

providing a window into the tissue microstructure.

DTI is traditionally employed to examine brain white matter health but can be used to assess changes in grey matter microstructure. Axonal organization and the lipid properties of myelin constrain water molecule motion helping to distinguish from a more diffuse water movement seen in the gray matter [64]. The two commonly reported indices derived from DTI are fractional anisotropy (FA) and mean diffusivity (MD). FA reflects the directionality of water diffusion and MD indicates an overall restriction of water diffusion. Decreases or increases in these measures, compared to normal, may suggest underlying neural pathology. Such sensitive indices as these will aide in uncovering the more subtle structural changes that precede severe atrophy.

While measures of anisotropy are classically reported in white matter, measures such as FA may also provide relevant information about gray matter organization. Measuring anisotropy applies in particular to structures with highly oriented fibers, such as the amygdala and hippocampus[65, 66]. As will be presented in my studies below, DTI may reveal changes to structures that are affected by AD concerning metabolic measures.

Each method provides a means of investigating the natural course of dementia due to AD. Each study utilizes a different measure to answer distinct questions that will inform the greater body of knowledge involving preclinical AD and mechanisms of metabolic risk factors.

Chapter 2: Insulin resistance and Cognition

Study 1: Midlife Insulin Resistance and Cognition: A longitudinal analysis

Introduction

Introduction

It in now widely accepted that insulin plays and important role in the CNS, including regulation of anabolic and catabolic processes. Evidence exists supporting the role of insulin in facilitating memory, promoting growth and survival of neurons and regulation of synapse receptor density (). Patients with AD exhibit impaired insulin signaling within the brain compared to those who did not develop dementia. For these reasons and more AD has been proposed to be a disease of underlying metabolic disturbances. Considering the effect of metabolism on overall cellular function, disturbances in metabolism may affect cognitive domains. When an individual presents to their physician, memory deficits are the primary cognitive complaints. However, it is accepted that other cognitive domains might deteriorate before significant memory changes. These cognitive domains include the executive function and speed and flexibility. Evidence suggests that insulin may affect cognitive domains associated with AD dementia. Further, effects of IR may initiate before significant cognitive deterioration is noticeable.

Midlife, in particular, appears to be a vulnerable life-stage about risk, with midlife cardiovascular risk factors predicting later cognitive decline and development

of AD[71]. DM2 patients suffer from cognitive impairment, lower mini-mental status exam and a lower rate of correct Clock Drawing Test {Mayeda, 2015 #101}. Epidemiological evidence reports an increased risk of developing dementia due to AD later in life**.** We poorly understand the mechanisms behind the cognitive decline observed in DM2 patients but are eager to comprehend the connections between midlife metabolic factors and dementia.

The molecular and cellular mechanisms underlying the increased risk of dementia in those with DM2 are continually investigated but poorly understood. Much focus is on the insulin resistance component of DM2. Insulin receptors are distributed throughout the cerebral cortex and concentrated in areas including olfactory cortex, frontal cortex, hypothalamus, amygdala, and the hippocampus[75]. For example, insulin is recognized as a key regulator of brain energy metabolism[72, 73]. While the brain does not require insulin to transport glucose into the cell for basal metabolic processes, evidence suggests that insulin is a primary regulator of molecular processes, particularly during tasks of learning and memory[74]. Animal studies demonstrate the involvement of insulin resistance and changes in cognitive function, particularly in the domain of learning and memory[76]. For example, animals that are insulin resistant take longer to find the platform in the Morris water maze test[77]. Research supports the presumption that insulin signaling is essential for normal cognitive function.

Population studies suggest insulin dysfunction affects both the energy homeostasis and may enhance the AD-associated pathology and cognitive

dysfunction[78]. Further, as noted above, conditions such as T2D are associated with increased risk for later development of dementia. What is unknown is whether or not midlife insulin resistance can predict long-term changes in cognitive abilities as measured by neuropsychological testing.

The following study aimed to assess the relationship between insulin resistance and cognitive slopes derived from longitudinal cognitive testing of non-demented, middle-aged participants. Primary, analyses centered around the question: What is the relationship between insulin resistance and longitudinal cognitive trajectories? The outcome measures reflected processes that are potentially affected by IR and/or AD and included: 1) the Mini-Mental State Examination (MMSE) a measure of global cognition, 2) the Wechsler Memory Scale-Revised (WMS-R) Logical Memory test to examine episodic memory, 3) Trails Making Test Part B (TMT-B), for assessing executive function, and 4) Trails Making Test Part A (TMT-A) as a measure of processing speed. I hypothesized that IR is with greater decline in cognitive function over time.

Methods

All study procedures were approved by the Health Sciences IRB of the University of Wisconsin-Madison.

Participants

Participants were chosen based on having completed at least three neuropsychological visits. One thousand and thirteen participants with a mean age at enrollment of 53 years old underwent a battery of cognitive tests. Further demographic information is listed in Table 1.

HOMA-IR

Insulin resistance was indexed with HOMA-IR ($\frac{fasting\ inuslinX\ fasting\ glucose}{405}$) using fasting blood glucose and insulin from each participant (described in more detail in Chapter 3, study 1). Seventy-six of the participants had identified themselves as having diabetes in the medical history questionnaire. We included those with diabetes to maintain the sample size. When the diabetics are removed from the model, significant results retain a p-value <.05 so we included the diabetics to add power to the study.

Cognitive assessment and cognitive slopes

Baseline and follow-up assessment of the participants included a battery of commonly used clinical neuropsychological tests[79], completion of questionnaires about health history and lifestyle, laboratory tests, and *APOE* genotyping.

Individual cognitive slopes were generated to provide a single value for each participant, for each cognitive test, with the slope reflecting cognitive performance over

time. The slopes were determined through linear mixed effects modeling in participants who had at least two data points (final slopes were generated from 2-5 visits, with a median of 3). Given that participants had slightly different timing between visits, slopes were adjusted and a test, re-test interval was calculated from the current age minus the baseline age.

Mini Mental Status Exam

The MMSE is a 30-point questionnaire used in clinical and research settings to screen for dementia. This exam includes orientation to time, orientation to place, registration, attention and calculation, recall, repetition and complex commands**[80, 81]**. The exam takes about 5-10 minutes to administer and is considered a measure of global cognition. The MMSE is typically interpreted using cut-offs to indicate levels of cognitive impairment, with a score of 24-30 reflecting normal cognition, 18-23 reflecting mild cognitive impairment, and 0-17 severe cognitive impairment. Because MMSE is screening tool, detailed cognitive evaluation beyond MMSE is needed to diagnose dementia[82].

Trail Making Test Parts A & B

The Trail Making Test (TMT) has two parts that test slightly different cognitive domains. TMT-A involves drawing a line connecting numbers sequentially. Performance reflects processing speed and is scored by the number of seconds it takes

to complete the task. Higher scores mean worse performance on the task. TMT-B is more cognitively complex and reflects executive function. For TMT-B participants are asked to connect a line alternating between numbers and letter. Better performance is reflected by faster time to completion. [83-86]

Wechsler Memory Scale-Revised (WMS-R) Logical Memory

A test administration involves an oral presentation of two short stories. For older adults, one story is presented twice[87]. Immediately after the end of the story presentation, the participant is asked to retell each story from memory. Episodic memory is assessed by asking the participant is to retell one or both stories following a delay[88].

Statistical Analysis

All analyses were conducted in SPSS v.22. Participants were included who had completed at least three visits. We conducted separate multiple linear regression models for each dependent cognitive variable of interest. The linear regression models tested the effects of insulin resistance on cognitive slopes, controlling for age at baseline testing, sex, test intercept, years of education and BMI. A follow-up test examined differences between high versus low HOMA-IR on cognitive slopes as modeled with ANCOVA. The Log_{10} HOMA-IR was binarized using a median split; participants were split into those that had HOMA-IR values less than or equal to the median (.23) and

those that had HOMA-IR values greater than the median. This new variable was used as a predictor while controlling for age at baseline testing, sex, test intercept, years of education and BMI. Graphs were plotted using the adjusted version of the dependent variable generated from running a regression with the covariates then saving the unstandardized residuals.

Results:

One thousand and thirteen participants were analyzed. A small percentage (~7%) had DM2. The average age of the sample was 53 years of age with the youngest 36 and the oldest 68 years. Our sample was predominately female, and the majority had a family history of AD. All participants cognitive testing scores fell within the normal range for each test.

Average HOMA-IR was 2.2. There were no significant results for insulin resistance predicting cognitive slopes of MMSE or TMT-B. Multiple linear regression analysis revealed a significant negative effect of HOMA-IR on TMT-A slopes, as shown in Table 2(β = .093, t(1007) = 2.73 p = .005). Modeled as an ANCOVA, the results showed that high HOMA-IR was associated with longitudinal decline on TMT-A compared to those below or equal to the median (β = .093, t(1010) = 2.73 p = .006)(Figure 2).

Table 1. Participant Demographics

N	1014
Age at intake	53.5 ± 6.5 (36-68)
Sex (Female)	69% (704)
BMI	28.79 ± 6.2 (17.5-56.5)
Family History Positive	73%(737)
Age at Lab draw	57.8 ± 6.5 (40-73)
HOMA-IR	2.2 ± 1.6
Log of HOMA-IR	.25 ± .28
Diabetic	7.5%(76)
Cognitive Slopes	
MMSE	-.0025 ± .31
TMT-A	-.139 ± .89
TMT-B	.196 ± 2.53
Logical Memory	-.0361 ± 1.53

Table 2. Multiple Linear Regression model for TMT-A N = 1013				
Dependent Variable	Predictor Variable	β	*t*	Sig.(p)
TMT-A slope	Intercept	-.570	-20.885	.000
	Age	.204	7.482	.000
	sex	-.042	-1.575	.116
	Years of education	-.013	-.474	.635
	BMI	-.023	-.701	.960
	Log_{10} HOMA-IR	.093	2.798	.005
Regression Model Statistics				
R^2 = .290 F(6,1007) = 74.874 *p*=<.0001				

FIGURE 1. INSULIN RESISTANCE EFFECTS ON TMT-A COGNITIVE SLOPES.

A scatter plot of the relationship between TMT-A after adjusting for age, sex, BMI, intercept and education years. Since TMT-A is scored on time to completion, the more positive scores indicate worse performances. Higher IR associated with worse decline that the low IR group

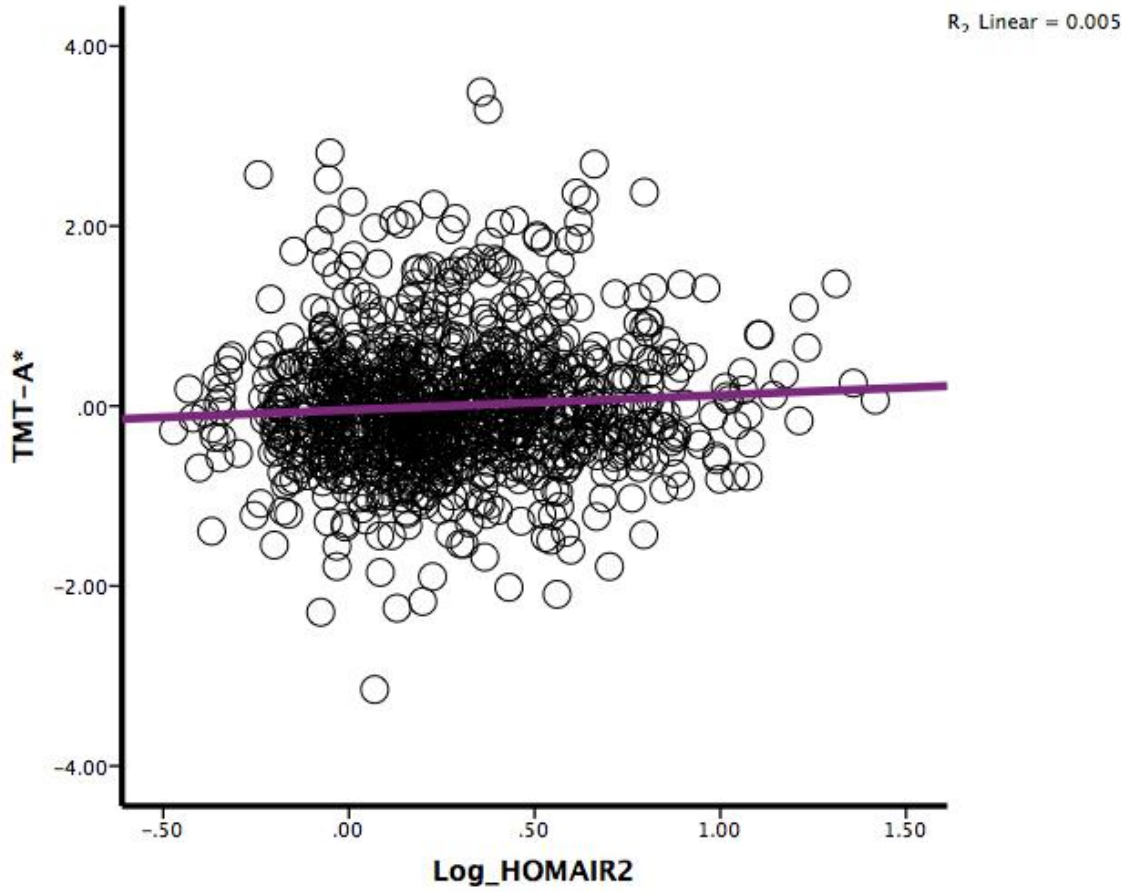
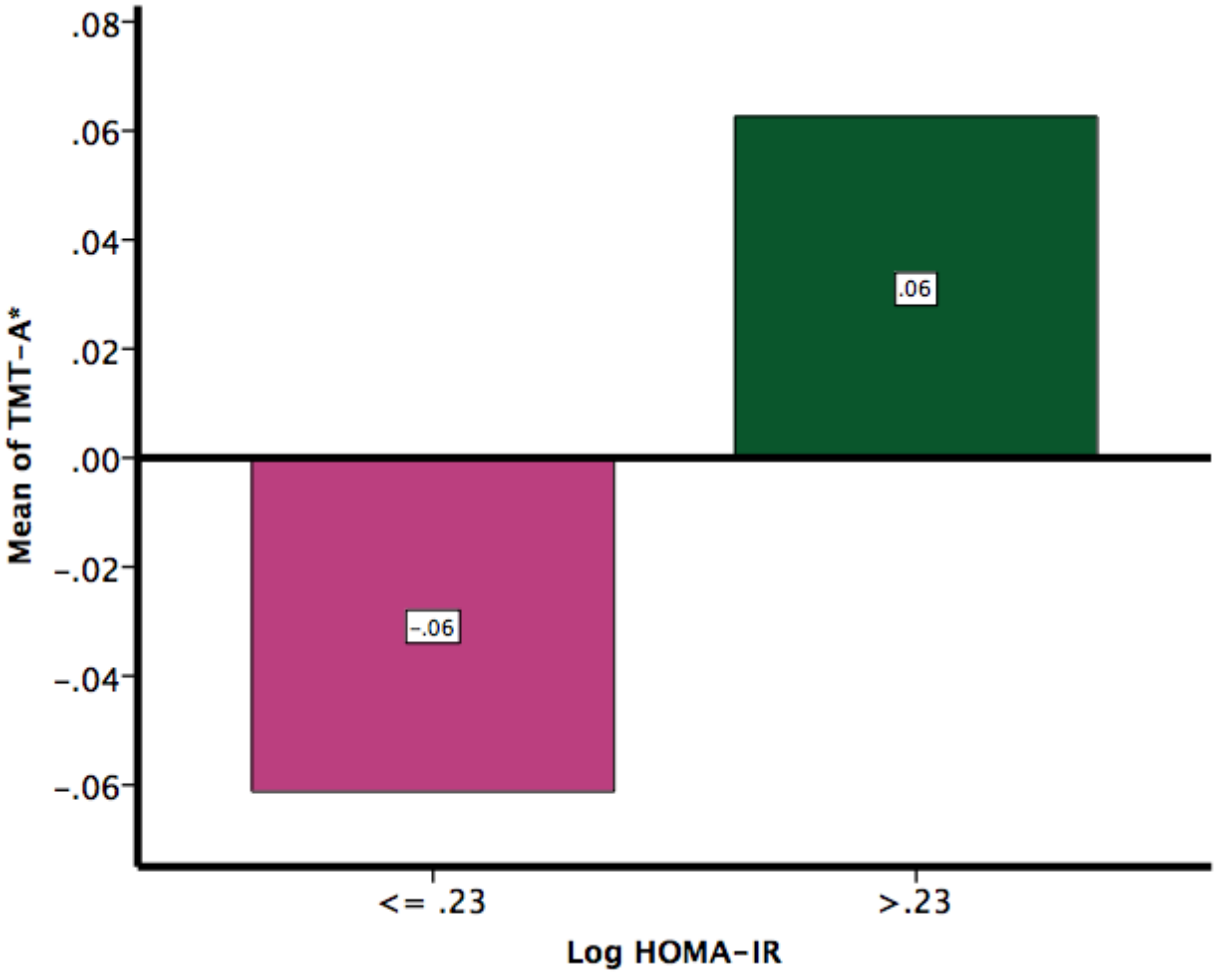


FIGURE 2. DIFFERENCES IN TMT-A PERFORMANCE BETWEEN MEDIAN SPLIT HOMA-IR GROUPS.

Bar graph showing the differences in average TMT-A after adjusting for age, sex, BMI, intercept and education years. Since TMT-A is scored on time to completion more positive scores, indicate worse performances. Thus, a more positive slope indicates increases in time to task completion over time. Plotted below are the slopes showing a more positive slope change for the high HOMA-IR group



Discussion

This analysis provides evidence for midlife insulin resistance predicting faster rates of cognitive decline. While I hypothesized that several domains would be affected, including memory and executive function, the analysis revealed that the cognitive domain affected by insulin resistance is processing speed. Although prior studies have shown that acute insulin administration increases memory function[34, 35, 89], it may be the case that insulin dysfunction is not severe enough in our participants to affect memory as measured by the neuropsychological testing.

There are several potential cellular processes underlying the cognitive abnormalities observed due to insulin resistance. Transgenic mice who have had insulin resistance induced by a high-fat diet, perform worse on tasks of memory than their insulin regular counterparts[90]. Also, these mouse models show decreased synaptic plasticity when an insulin-resistant state is generated by streptozocin treatment[77]. Alterations to synapses could cause reduced speed of processing while maintaining other facets of cognitive function[91]. While processing speed tends to decline with age[92], this analysis controlled for age, indicating that insulin resistance at midlife may cause a reduction that is above and beyond the effect of age.

Alzheimer's disease (AD) is defined by the accumulation of amyloid plaques and neurofibrillary tangles, which begin depositing before clinical symptoms emerge[67, 68]. Amyloid plaques, extracellular accumulations of APP cleavage, and tangles, intracellular accumulation of misfolded tau protein, are believed to interfere with

neuronal function, consequently leading to the development of significant cognitive impairment and dementia[69, 70]. However, amyloid plaques and NFT take many years to accumulate, and amyloid plaques, in particular, correlate poorly with cognitive impairment.

This study provides preliminary evidence of insulin resistance at midlife leading to a long-term cognitive consequence. A strength of this analysis is the large sample size (>1000) and longitudinal nature of the study. However, this study does not address the mechanism by which insulin resistance may affect cognitive decline. Prior studies from our laboratory suggest that cerebral glucose metabolism may play a role, or that amyloid deposition may be involved[78]. The next chapters will focus on using biospecimens and imaging techniques to explore in-vivo effects of insulin resistance on neural microstructure and accumulations of AD pathology.

Chapter 3 Insulin, Obesity and CSF biomarkers

Study 2: Insulin Resistance and markers of Amyloid

Introduction

Mounting epidemiological evidence suggests Diabetes Mellitus is associated with and increased a risk of dementia due to Alzheimer's disease (AD)[93-95]. Much focus is on the role of insulin resistance (IR) in increasing the risk for AD [96-98]. Insulin receptors are distributed throughout the brain in regions including hippocampus, amygdala and frontal lobes, significant territories for AD pathology accumulation [99]. Interestingly, insulin receptor density decreases in aging; Insulin signaling reduced in AD [100, 101] and post-mortem brain tissue from AD patients shows decreased insulin mRNA compared to controls [98]. Additionally, the prevalence of impaired fasting glucose and type 2 diabetes is increased in AD patients compared to age-matched controls [102]. Studies suggest insulin signaling may be a key factor in modulation of AD pathology. Some human studies suggest IR and type 2 diabetes are linked with abnormal phosphorylation of tau protein[103] and greater amyloid burden[23], although other studies suggest otherwise [104-106]. We need a better understanding of the possible consequences of IR at midlife especially in people who are already positive for other AD risk factors such as carriers of the APOE4 ε4 allele or a parental family history of AD.

Insulin resistance has many implications in AD especially in regards to Aβ processing and amyloid formation. In an insulin resistant state there is less response from insulin receptors, thus signaling does not transfer from the extracellular to the intracellular signaling cascade. Insulin resistance can modulate the proteolytic processing of amyloid precursor protein (APP) into its cleavage products which include

Aβ peptides[36, 107]. Generally, APP is processed by one of two pathways: the β-secretase-mediated or the α-secretase-mediated cleavage pathway. Both pathways produce multiple end products, but only the β-secretase-mediated pathway generates the amyloidogenic cleavage products of APP processing, Aβ peptides[108, 109]. Cleavage of APP by α-secretase is believed to lessen the formation of extracellular amyloid plaques by avoiding the formation of amyloid beta (Aβ) and producing soluble peptides and the secreted APP-alpha (sAPPα) [110]. In the β-secretase pathway cleavage by γ-secretase adjacent to residue 42,40 or 38 is the last step in generating the peptide of variable lengths and the secreted APP beta[111]. The secreted form of APP has been shown to have differential effects on brain health. The alpha form has neuroprotective properties and contributes to overall brain health while the beta form lacks these features, one of the reasons why the α-secretase pathways are thought of as more favorable [112, 113]. $A\beta_{42}$ is believed to be the most toxic form of Aβ because it has the closest correlation with amyloid deposition. However, the other forms are also found in amyloid plaques[114]. By examining multiple forms of Aβ, one can derive information about the production and processing of amyloid.

Insulin's role in APP processing has been explored in-vivo and in-vitro. Transgenic mouse models of AD mice, IR promotes the generation of $A\beta_{42}$ peptides and amyloidosis[20, 115], part of a pathway instigated by cleavage of amyloid precursor protein by β-secretase. IR is also linked with amyloidosis by increasing γ-secretase activity and decreasing insulin degrading enzyme (IDE) activity[20]. IDE regulates Aβ

levels in neuronal cells, and IDE knockout mice have hyperinsulinemia, glucose intolerance, and increased cerebral accumulation of Aβ[22]. Diabetic phenotypes in mice are also associated with increased tau phosphorylation[116]. In-vitro studies have also shown a general reduction in Aβ due to decreased production of APPβ and increased movement toward the α-secretase pathway leading to increases in sAPPα in the cell serum[117]. Clinical analysis has also shown an association between insulin administration and lower plasma APP levels in normal older adults[37]. The cerebrospinal fluid bathes the brain, and many of the products are cleared in the CSF and can be measured as biomarkers of processes occurring more specifically in the brain.

Much of the work assessing diabetes, obesity, insulin, and AD pathology has been completed in animal models. There is a need for translation of these findings to human studies. The purpose of this work is to assess the consequences of midlife insulin resistance on the deposition of amyloid and APP cleavage products in the CSF in individuals at an increased risk of developing AD. Specifically, we examined the effects of IR and *APOE*ε4 status on CSF sAPP, sAPP-β, $A\beta_{42}$, $A\beta_{40}$ $A\beta_{38,}$ and $A\beta_{42}/A\beta_{40}$ taking into account the effect of APOE4 and family history on CSF levels of amyloid markers. We hypothesized that both IR and *APOE*ε4 would be associated with decreased markers of amyloid.

Methods:
Participants.

One hundred and eleven cognitively healthy late-middle-aged adults (mean age = 60.4 years, range= 47-75 years, 69% women) from Wisconsin Registry for Alzheimer's Prevention (WRAP) were included in the sample. The WRAP study is focused on collecting data related to risk factors at middle age including genetic, family history of AD and biological factors postulated to contribute to the development of Alzheimer's dementia. Inclusion criteria for this study consisted of no clinical diagnosis of a memory disorder, no current diagnosis of major psychiatric disease or other major medical conditions (e.g., myocardial infarction, or recent history of cancer), and no history of head trauma. Participants were also required to have available fasting insulin and glucose levels, in addition to CSF assays for AD-relevant biomarkers. After considering all of these parameters the sample size was 115 and when outliers were excluded, the sample comprised one hundred and twelve participants.

Parental family history (FH) of AD classification was based on probable or confirmed AD of one or both parents [118]. A positive family history was defined as having one or both parents with autopsy-confirmed or probable AD as outlined by research criteria [15, 16], reviewed by a multidisciplinary diagnostic consensus panel. Detailed medical history and phone interviews were conducted to confirm the absence of parental FH. A negative family history is based on if the participant's father survived to at least age 70 and the mother lived to age 75 without incurring a formal diagnosis of

dementia or exhibiting cognitive deterioration. Participants were classified using a binary variable as FH positive(1) or FH negative(0).

Venous blood glucose and insulin were extracted the morning after a 12hr fast and collected in 9mL polypropylene tubes clotted for 30min then centrifuged at 4*C at 3000 rpm for 10mins. Cells and plasma separated and plasma was collected and aliquoted into 1.5mL microcentrifuge tubes and frozen at -80 degrees Celsius. Plasma and serum samples were analyzed at the University of Wisconsin Hospital and Clinics Hospital Laboratory (Madison, WI). Plasma was assayed using glucose hexokinase method (Siemens Dimension Vista) and chemiluminescent immunoassay on an ADVIA Centaur XP Immunoassay System (Siemens Corporation, Washington DC, USA) to assess fasting glucose and fasting insulin, respectively. Insulin resistance was measured with HOMA-IR ($\frac{fasting\ insulin\ X\ fasting\ glucose}{405}$) using fasting blood glucose and insulin from each participant.

HOMA-IR was analyzed as a continuous variable. Because the raw data were skewed, all analyses used $\log_{10}$ transformed HOMA-IR. The presence of type 2 diabetes in this sample was assessed by reviewing medication records, self-report, and using American Diabetes Association criteria, where participants with fasting blood glucose over 125 mg/dL were flagged as diabetic and excluded from the analysis to focus on pre-diabetes only. There were two diabetics in total. Also, one participant had an HOMA-IR value greater than three standard deviations from the mean and was also excluded from the analysis. Exclusion criteria left us with 111 participants.

Body mass index (BMI) was derived from weight and height (measured without shoes). Weight was measured to the nearest tenth of a kilogram using a medical scale balance beam model, Health-O-Meter. Height was measured to the nearest centimeter using a wall-mounted ruler.

Lumbar Puncture

A gentle extraction technique was employed to collect CSF via lumbar puncture using a Sprotte 25- or 24-gauge spinal needle at the L3/4 or L4/5 level of the spine. Each participant had approximately 22mL of CSF extracted and centrifuged at 2000g for 10mins. Samples were frozen in polypropylene tubes in 0.5mL aliquots and stored at -20 degrees for at least 24hr then transferred to the -80 degrees Celsius by a trained graduate student.

Cerebrospinal fluid was sent to Sweden for analysis where board-certified laboratory technicians blinded to clinical information analyzed all samples by protocols approved by the Swedish Board of Accreditation and Conformity Assessment (SWEDAC). CSF levels of Aβ38, Aβ40, and Aβ42 levels were determined using Aβ triplex assay (Human Aβ peptide Ultra-Sensitive Kits) developed by Meso Scale Discovery, Gaithersburg, Maryland, USA as previously described extensively[21]. Briefly, this assay uses C-terminus specific antibodies to detect the various forms of Aβ peptides and an SULFO-TAG^{UM} labeled anti-Aβ antibody for detection with electrochemiluminescence. CSF levels of sAPPbeta and sAPPalpha were assayed using commercially available enzyme-

linked immunosorbent assay (ELISA) methods (INNOTEST assays, Fujiurebio, Ghent Belgium) as previously described in detail (Palmqvist et al., 2014).

*APOE*ε(2,3,4) alleles were determined using genetic testing performed in the UW Hospital laboratory. Non-fasting blood samples collected at the baseline visit were genotyped with standard polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing. Once extracted the DNA was genotyped with the use of a homogenous Florescent Resonance Energy Transfer technology coupled to competitive allele-specific PCR (LGC Genomics; Beverly, MA). APOE4 carriers were coded as 1 for carriers and 0 for non-carriers.

Statistical Analysis

Multiple linear regression models were implemented in SPSS23 to assess the effect of HOMAIR on the amyloid biomarker levels of the participants (sAPP, sAPP-β, $A\beta_{42}$, $A\beta_{40}$ $A\beta_{38,}$ and $A\beta_{42}/A\beta_{40}$). Covariates included age at lumbar puncture visit, sex, and BMI. Outliers were determined during quality control. Using SPSS, outliers were defined as greater than three standard deviations above the mean for each variable. **Age** at the lumbar puncture visit was determined by subtracting each participant's date of birth from the date of his or her lumbar puncture visit. Each dependent variable derived from CSF was tested separately. All of the covariates were chosen as they also gave effects on AB deposition or clearance and we were interested in the effects of IR while controlling for other known risk factors.

Results

Demographics

Demographics and clinical characteristics of the participants are shown in Table 1. One hundred and eleven participants underwent blood collection, lumbar puncture neuropsychology testing. All of the participants were cognitively normal as determined by MMSE scores in the normal range (29.39 ± .78). Demographics report a majority of women(69%) and those with an FH(78%) of AD. All of the participants, except one, had normal glucose and insulin measures. Currently, a pathological cutoff for HOMA-IR does not exist. Thus, we considered HOMA-IR as a continuous variable.

Table 3. Summary of Multiple Regression Analysis for CSF Aβ1-42 (N = 111)

N	111
Age	60.4 ± 5.9 (47-75)
Gender (Women)	69% (77)
BMI	28.4 ± 5.2 (18.9-41.7)
Fasting plasma Glucose	94.3 ± 9.91 (74-132)
Fasting Plasma Insulin	9.38 ± 9.02 (2-31)
Log of HOMA-IR	.25 ± .27
APOE ε4	37% (41)
Family History Positive	78% (87)
MMSE	29.39 ± .78
Education (years)	16.64 ± 2.44
Cerebrospinal Fluid (pg/mL)	
$A\beta_{1-42}$	
$A\beta_{1-40}$	1048.2 ± 348.55
$A\beta_{1-38}$	10949.0 ± 2908.6
sAPPα	1674.2 ± 477.2
sAPPβ	645.8 ± 281.7
	538.8 ± 217.0

Insulin Resistance and amyloid precursor protein

One of the participants had missing values for sAPP, so they were not included in this analysis. Results of the multiple regression analyses showed HOMA-IR is negatively associated with CSF sAPPβ (β= -0.297, t(109)= -2.436, p= 0.017), and sAPPα (β= -0.331, t(109)= -2.727, p= 0.008). APOE4 also had a significant effect in the model. BMI was not associated with the sAPP peptides.

FIGURE 3: SCATTERPLOTS SHOWING ASSOCIATION BETWEEN LOG10 HOMA-IR AND CSF APP BIOMARKERS

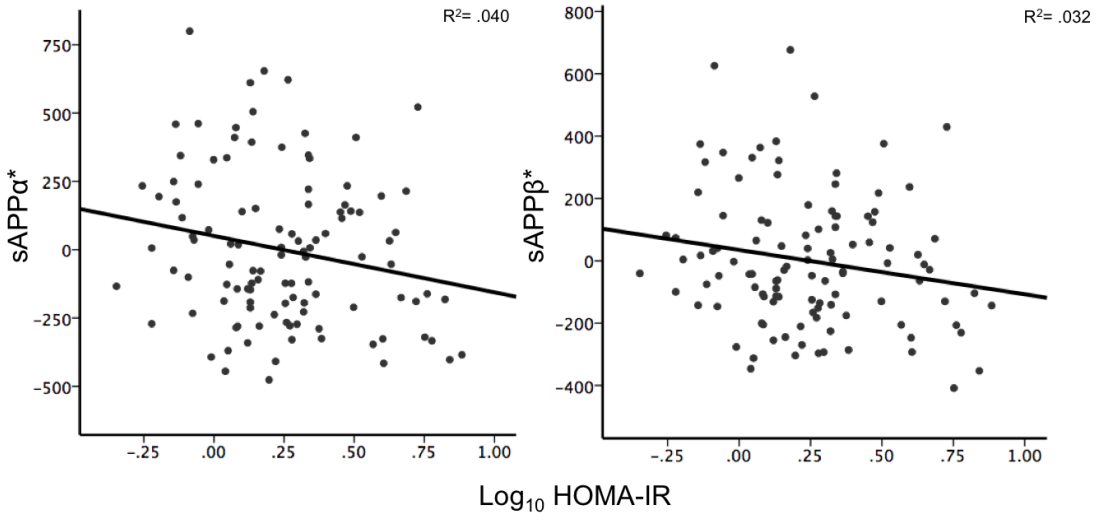


FIGURE 3: SCATTERPLOTS SHOWING RELATIONSHIP BETWEEN LOG_{10} HOMA-IR AND CSF APP BIOMARKERS

Insulin Resistance, BMI and CSF Aβ peptides

Results of the multiple regression analyses showed HOMA-IR is negatively associated with CSF $A\beta_{38}$(β= -.410, t(110)= -3.499, p= 0.001) (Table 2), $A\beta_{40}$(β= -.390, t(110)= -3.351, p= 0.001) (Table 3), and $A\beta_{42}$(β= -.313, t(110)= -2.676, p= 0.009)(Table 4).

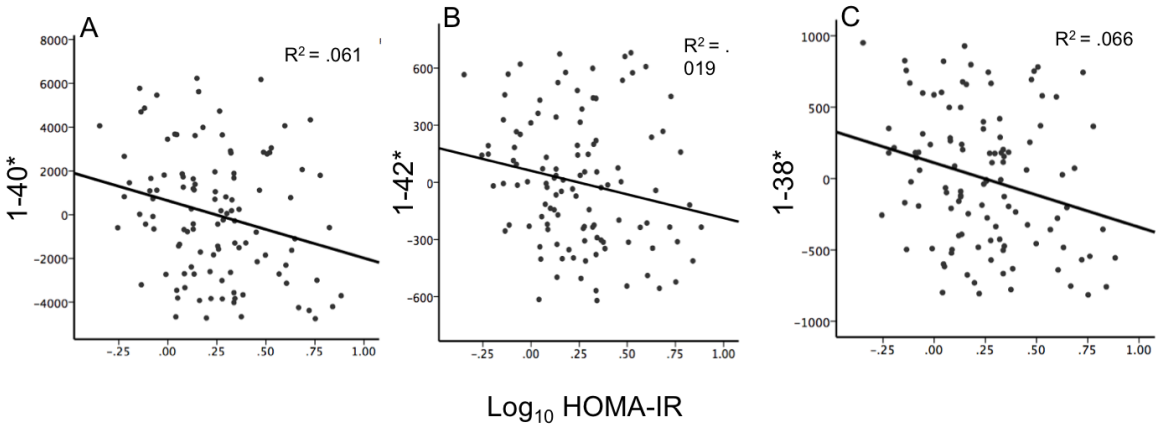
Additionally, our model showed BMI to be predictive of changes in CSF Aβ. Interestingly, the effect is in the opposite direction of insulin resistance, with increases in BMI associated with increases in $A\beta_{38}$(β= 0.307, t(110)= 2.615, p= 0.010), $A\beta_{40}$(β= 0.288, t(110)= 2.465, p= 0.015), $A\beta_{42}$(β= 0.305, t(110)= 2.655, p= 0.009). The BMI findings are important as obesity at midlife has been shown to increase the risk of late-onset AD development.

Table 4. Summary of Multiple Regression Analysis for CSF Aβ1-42 (N = 111)				
Dependent Variable	Predictor Variable	β	*t*	Sig.(p)
Aβ1-42	Age	-.031	-.314	.696
	Gender	-.030	-.311	.564
	Family History	-.115	-1.205	.185
	APOE ε4	-.177	-.1.887	.062
	BMI	.309	2.640	.012*
	Log_{10} HOMA-IR	-.314	-2.681	.009*
Regression Model Statistics				
R^2 = .115 F(7,105) = 2.260 *p*=.043				

Table 5. Summary of Multiple Regression Analysis for CSF Aβ1-40 (N = 111)				
Dependent Variable	Predictor Variable	β	*t*	Sig.(p)
Aβ1-40	Age	.139	1.416	.160
	Gender	-.002	-.020	.984
	Family History	-.121	-1.272	.206
	APOE ε4	-.051	-.547	.585
	BMI	.293	2.519	.013*
	Log_{10} HOMA-IR	-.398	-3.395	.001*
Regression Model Statistics				
R^2 = .126 F(7,105) = 2.497 *p*=.027				

Table 6. Summary of Multiple Regression Analysis for CSF Aβ1-38 (N = 111)				
Dependent Variable	Predictor Variable	β	*t*	Sig.(p)
Aβ1-38	Age	-.089	.903	.174
	Gender	-.043	-.447	.656
	Family History	-.060	-.625	.534
	APOE ε4	-.026	-.278	.781
	BMI	.309	2.640	.012*
	Log_{10} HOMA-IR	-.314	-2.681	.009*
Regression Model Statistics				
R^2 = .116 F(7,105) = 2.267 *p*=.043				

FIGURE 4: SCATTERPLOTS SHOWING ASSOCIATION BETWEEN LOG$_{10}$ HOMA-IR AND CSF Aβ BIOMARKERS



Discussion

This study showed that among a middle-aged cohort of cognitively intact individuals enriched for AD risk factors, insulin resistance predicts decreased CSF sAPPα and β and decreased Aβ42, Aβ40, Aβ38 while having no significant effect on the pathology related ratio of Aβ42/Aβ40. Our study suggests that mechanisms leading to increased AD pathology begin in middle age before the onset of dementia symptoms. Consistent with previous literature in amyloid models, our study demonstrates that insulin resistance predicts a decline in CSF amyloid biomarkers. Further, our regression models revealed that body mass index is also predictive of CSF AB but in the opposite direction from the effects of insulin resistance. Our findings suggest insulin may

decrease the production of sAPP and the cleavage products while increased body mass may increase clearance mechanisms.

Insulin resistance has a role in deposition and clearance of amyloid plaques. Animal models demonstrate increased amyloid in the brain of AD transgenic mouse with diet-induced insulin resistance [119], as well as increases in Aβ42, Aβ40 peptides corresponding to the increases in amyloid. Another study demonstrated substantial effects of insulin on the clearance of Aβ40[34]. In human research, an analysis of brains from the Hiyasama study revealed an increased odds ratio for developing neuritic plaques [31]with effect sizes that were larger than those observed for the *APOE ε4* allele. Further, Willete et al. found increased amyloid deposition as measured by PIB-PET imaging in middle–aged cognitive asymptomatic individuals with higher HOMAIR[120]. Furthermore, brain tissue from AD patients shows fewer insulin receptors, less insulin mRNA, and c-peptide suggesting increased insulin dysfunction in those with AD[121].

One of the strengths of this study is the focus on several markers of amyloid. Most studies have focused on one or two biomarkers of amyloid separately, while we show here that insulin resistance affects all amyloid biomarkers, including upstream cleavage products of the precursor protein. From these findings, a global decrease in amyloid associated peptides may imply a more upstream role for insulin signaling in APP processing. The idea that insulin may affect the APP processing is supported by several experimental observations. Insulin affects the production of APP and its

cleavage products. Cellular studies have demonstrated insulin's ability to modulate intracellular APPβ and Aβ levels. Evidence for insulin's modulation of APP is further supported by studies showing a dose response effect of insulin administration or digestion of glucose on the levels of APP[122]. This modulation may vary in the course of insulin resistance as there may be increased insulin signaling at the beginning of the disease which is later decreased through unresponsive insulin receptors.

Decreases in CSF peptide may indicate abnormal clearance levels in our participants due to disrupted insulin signaling. Research investigating Aβ clearance mechanisms have identified insulin-degrading enzyme (IDE), *a* key enzyme which is also a significant removal mechanism for insulin degradation [22, 123-125]. Insulin's role in increased amyloid deposition is thought to be due to competitive binding between insulin and Aβ. In IR there is excess insulin, which has a much higher binding affinity for IDE than Aβ[126]. Thus, the occupation of IDE by the excess insulin decreases the availability of IDE for Aβ clearance. The reduced breakdown and clearance would be reflected as decreased deposition into the cerebrospinal fluid. Evidence suggests, decreases in CSF Aβ reduced with increased amyloid deposition in the brain parenchyma[127]. Insulin's exacerbation of amyloid pathology is becoming clearer as research continues and it is reassuring to find results in our human studies that converge with in-vivo and in-vitro analyses.

These studies provide information about the possible effects of insulin and overall body mass on the production and deposition of Aβ. Here we find that higher insulin resistance is associated with decreases in Aβ in the CSF.

Insulin resistance may also interfere with APP processing at the enzymatic level. Transgenic insulin resistant mice show decreased insulin receptor signaling as well as decreased activity of β and α secretase. Importantly, the observation that all measured amyloid markers were decreased with higher insulin resistance suggests: 1) there is increased cerebral deposition of all forms of amyloid in the presence of higher insulin resistance, or 2) there is a marked decrease in the APP processing pathway leading to decreased cleavage products. Evidence supporting decreased cleavage comes from a neuronal insulin receptor knockout mouse, where decreased α- and β-secretase activity, as measured by assay of hippocampal lysates, are observed [128]. This decline in secretase activity led to an overall reduction of APP cleavage products in the brains of these insulin-resistant mice. It is possible this same mechanism is occurring in the human brain. More specifically, insulin signaling is thought to be a regulator of β and α secretase activity through the PI3-K activity, a downstream target of insulin receptor signaling. Insulin's importance in this pathway has also been showing through mobilization of the Aβ and APP from the intracellular trans-Golgi network, a region where amyloid is assembled if not mobilized [113]. Following this reasoning, insulin resistance could lead to decreased mobilization out of the cell compounded with decreased clearance through IDE resulting in decreased levels of Aβ in the CSF.

Finding the positive association between BMI and CSF Aβ is consistent with results from a 2011 study assessing BMI and CSF Aβ, which found that BMI is associated with increased CSF Aβ and decreased amyloid deposition as determined by positron emission tomography scanning with PIB[129]. These studies were conducted in a group that included AD and mild cognitive impairment as well as non-demented older adults. Our study implies these mechanisms may be at play even before a clinical diagnosis of AD. Increases in body mass also correlate with increased secretion of adipokines; hormones secreted from adipose tissue. Of particular interest is the hormone leptin[130]. Leptin is a peptide hormone implicated in satiety and is released into the bloodstream at the same time as insulin[131, 132]. Leptin has been shown to increase the clearance of Aβ, which in turn could account for the increase in apparent CSF Aβ[133]. While we do not have a measure for leptin, our findings are consistent with increased CSF levels of Aβ in those with higher BMI. The result also implies that controlling for BMI is important in studies of IR, given that these two metabolic risk factors correlate with one another but may have differential effects on CSF biomarker.

Finally, this study provides significant additional evidence of examining brain changes in midlife. This study suggests that metabolic factors including insulin and obesity affect cognition and the processing of APP peptides into cleavage products. Further, insulin resistance seems to have the largest effect on the Aβ isoforms less associated with amyloidogenesis. Abnormal insulin signaling may lead to increased

production as well as deposition into the brain parenchyma. Further studies analyzing PIB deposition at middle age can enhance the validity of these findings in our cohort.

In conclusion, metabolic factors such as insulin resistance and obesity have an impact on amyloid biomarkers at midlife. This study demonstrates for the first time that pre-diabetic levels of IR predict a linear decline of CSF Aβ in a late-middle-aged cohort while BMI predicts increases.

Study 3: Insulin Resistance and markers of neurofibrillary tangles

N.B: These data are published in part in Starks et al. Insulin Resistance is Associated with Higher Cerebrospinal Fluid Tau Levels in Asymptomatic APOEε4 Carriers. J Alzheimer's Dis. 2015;46(2):525-33.

INTRODUCTION

The majority of studies focused on type 2 diabetes and insulin resistance (IR) in relation to AD have focused on mechanisms related to amyloid [24, 115], [24]. [22], however, diabetic phenotypes in mice are also associated with increased tau phosphorylation[116], and human studies also suggest IR and type 2 diabetes are linked with abnormal phosphorylation of tau protein[103].

In order to examine the contribution of IR to development of NFT pathology at the critical juncture of midlife and presumed preclinical phase of AD, this study examined the relationship between IR and cerebrospinal fluid (CSF) markers of tau pathology in asymptomatic late-middle-aged adults with risk factors for AD. As noted above, CSF biomarkers show utility for predicting conversion to AD [55] and provide an indirect, but close marker of tissue pathology [134, 135]**.** Two common markers of tau pathology include phosphorylated tau (P-tau$_{181}$) and total tau (T-tau), both of which are elevated in mild cognitive impairment (MCI) and AD [136] [137].

In order to examine the relationship between IR and tau pathology in the preclinical phase of AD, data were examined from participants in the Wisconsin Registry for Alzheimer's Prevention (WRAP) study, a cohort enriched for AD risk factors, including *APOE* ε4 genotype (40%) and parental family history of AD (75%). Based on evidence from animal models and human epidemiological studies suggesting IR contributes to AD risk and pathology, we hypothesized that higher IR would be

associated with higher P-Tau$_{181}$ and T-Tau. Additionally, we examined the ratio of both of these markers to Aβ42.

METHODS

Participants. This study examined 113 asymptomatic late-middle-aged adults (mean age = 60.6 years, range= 47-75 years, 70% female) from WRAP who had undergone lumbar puncture and fasting blood draw. This continuing study assesses genetic and biological factors postulated to contribute to the development of dementia-related cognitive decline and neural dysfunction. Inclusion criteria for this study entailed no clinical diagnosis of a memory disorder, no current diagnosis of major psychiatric disease or other major medical conditions (e.g., myocardial infarction, or recent history of cancer), and no history of head trauma. Participants were also required to have available fasting insulin and glucose levels, in addition to CSF assays for AD-relevant biomarkers.

Parental family history (FH) of AD classification was based on probable or confirmed AD of one or both parents [118]. A positive family history was defined as having one or both parents with autopsy-confirmed or probable AD as outlined by research criteria [138, 139], reviewed by a multidisciplinary diagnostic consensus panel. Detailed medical history and phone interviews were conducted to confirm absence of parental FH, where absence required that the participant's father survive to at least age 70 and the mother to age 75 without incurring a formal diagnosis of dementia or exhibiting cognitive deterioration. Participants were classified using a binary variable as FH positive or FH negative.

Fasting blood samples were collected following vital sign measurement and processed at the University of Wisconsin Clinical Research Unit and fasting glucose and insulin values were determined at the UW Hospital laboratory. Subjects' weight and height were measured without shoes. Weight was measured to the nearest tenth of a kilogram using a medical scale balance beam model, Health-O-Meter. Height was measured to the nearest centimeter using a wall-mounted ruler. Body mass index (BMI) was calculated based on height and weight.

Insulin resistance was measured by HOMA-IR (glucose*insulin/405) using fasting blood glucose and insulin from each subject. Currently, cut-off points of HOMA-IR for insulin resistance are not well established. For example, studies in American Indians, American Mexican, Spanish, Japanese, and European populations demonstrate great variability in HOMA-IR cut-offs and cut points have not been determined for a sample comparable to ours [140-143]. Thus, HOMA-IR was analyzed as a continuous variable. Because the raw data were skewed, all analyses used log transformed HOMA-IR. Presence of type 2 diabetes in this sample was assessed by reviewing medication records, self-report, and using American Diabetes Association criteria, where participants with fasting blood glucose over 125 mg/dL were flagged as diabetic. Four participants had previously been diagnosed with type 2 diabetes and were taking Metformin. One participant not diagnosed with diabetes had elevated fasting glucose according to ADA criteria. Medications were also reviewed to identify additional participants with diabetes. In order to test for relationships in the pre-diabetic range, the

analyses were repeated excluding participants with diagnosed or possible diabetes. The results did not differ, thus, these 4 participants were retained.

APOE ε4

APOE ε4 extraction and isoform classification have been described previously [144]. Participants were categorized using a binary variable as being non-carriers (zero ε4 alleles) or *APOE* ε4 carriers (one or two ε4 alleles).

Cerebrospinal Fluid

CSF was collected in the morning after a 12hr fast with a Sprotte 25-or 24-gauge spinal needle at the L3/4 or L4/5 using gentle extraction into propylene syringes. CSF (~22mL) was then combined, gently mixed and centrifuged at 2000g for 10 minutes. Supernatants were frozen in 0.5mL aliquots in polypropylene tubes and stored at -80^{o}C. Samples were analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) methods (INNOTEST assays, Fujiurebio, Ghent Belgium) as described previously in detail [145]. Board-certified laboratory technicians who were blinded to clinical information performed all analyses. All samples were analyzed according to protocols approved by the Swedish Board of Accreditation and Conformity Assessment (SWEDAC) using one batch of reagents (intra-assay coefficients of variation <10%).

Statistics

A multiple regression analysis was conducted using SPSS (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) software to examine the effects of

HOMA-IR, *APOE* ε4 genotype and FH status on P-Tau$_{181}$, T-Tau and Aβ$_{42}$. Separate analyses were performed for each CSF measure of interest. A secondary analysis also tested the effect of IR on Aβ$_{42}$/P-Tau$_{181}$ a ratio that takes into account both facets of pathology with a smaller ratio implying higher AD pathology[146]. Interactions between HOMA-IR and APOE ε4 genotype, and HOMA-IR and FH status were also examined. Covariates were age, sex and BMI.

Outliers and collinearity. BMI and IR are positively correlated with one another, which could lead to collinearity artifacts in the regression analyses. We assessed for collinearity effects of BMI and the log of HOMA-IR by calculating the variance inflation factor (1.5), which revealed that the variables were not highly co-linear. Our original N was 115, however two participants were identified as significant outliers (> 3 SD from the mean) on CSF Aβ$_{42}$ (one participant) and HOMA-IR (one participant), even after log transforming HOMA-IR, and thus were removed from further analysis for a total N of 113.

RESULTS

Demographics

Demographics and clinical characteristics of the participants are shown in Table 7. Mean MMSE across all participants was well within the normal range (29.36 ± .80). Mean HOMA-IR was 2.2 (SD = 1.6), with untransformed values ranging from 0.45-7.65.

Table 7. Participant Characteristics				
		APOE ε4		
	All	Carrier (38%)	Non-carriers (62%)	*p*
N	113	43	70	
Age	60.5 ± 5.9 (47-75)	60.5 ± 6.2	60.4 ± 5.8	.923
Sex (Female)	69% (78)	77%(33)	64% (45)	.164#
BMI	28.5 ± 6.0 (18.9-52.7)	29.0 ± 5.9	28.12 ± 4.8	.378
Fasting plasma Glucose	95.3 ± 10.6 (74-132)	93.98 ± 10.5(74-124)	95.96 ± 10.2	.325
Fasting Plasma Insulin	9.38 ± 7.2	10.41 ± 8.9	9.0 ± 5.9	.225
Family History Positive	77%(89)	82%(36)	75%(53)	.312#
HOMA-IR	2.2 ± 1.6	2.1 ± 1.4	2.2 ± 1.6	.941
Log of HOMA-IR	.25 ± .28	.28 ± .30	.24 ± .27	.437
Cerebrospinal Fluid (pg/mL)				
$A\beta_{1-42}$	756.2 ± 222.5	703.36 ± 199.5	769.0 ± 231.0	.092
P-Tau_{181}	42.9 ± 13.9	43.2 ± 14.4	42.63 ± 13.6	.842
T-Tau	318.6 ± 112.3	331.9 ± 104.2	310.34 ± 116.9	.326
$A\beta_{1-42}$ / P-Tau_{181}		.067	.059	.162
# Chi-square				

Effects of HOMA-IR on P-Tau$_{181}$, T-Tau and Aβ_{42}

A multiple regression analysis was conducted to test the effect of HOMA-IR on Aβ_{42}, T-Tau, and P-Tau$_{181}$ including the covariates of sex, age, BMI, FH, *APOE* ε4 status, HOMA-IR, and the interaction of *APOE* ε4 status and HOMA-IR. Each CSF measure was tested individually as a dependent variable in a multiple regression analysis. There was no main effect of HOMA-IR on the CSF markers.

Our regression model indicated a significant interaction between *APOE* ε4 and HOMA-IR on CSF P-tau$_{181}$ after adjusting for covariates, (β= 0.369, t(112)= 2.422, p= 0.017) where there was a positive relationship between HOMA-IR and P-Tau$_{181}$ among *APOE* ε4 carriers, and a negative relationship between HOMA-IR and P-Tau$_{181}$ among non-carriers (Figure1, Table 2). Likewise, there was a significant interaction between HOMA-IR and *APOE* ε4 status on CSF T-Tau (β= 0.367, t(112)= 2.391, p= 0.002), whereby there was a positive relationship between HOMA-IR and T-Tau among *APOE* ε4 carriers, but HOMA-IR was negatively associated with T-Tau$_{181}$ among *APOE* ε4 non-carriers (Figure 2, Table 3). There was no significant effect of HOMA-IR, *APOE* ε4, or the interaction term on CSF Aβ_{42} alone.

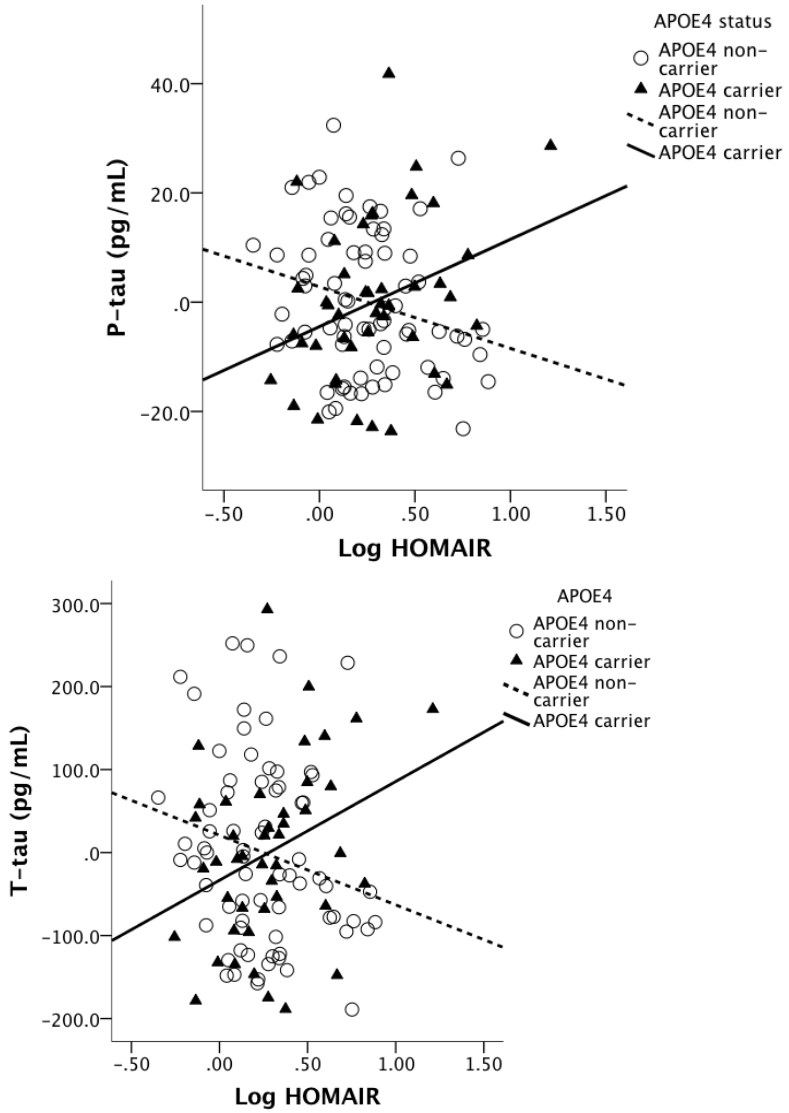
Table 8. Summary of Multiple Regression Analysis for CSF P-Tau$_{181}$ (N = 113)						
Dependent Variable	Predictor Variable	B	SE(B)	β	*t*	Sig.(p)
P-Tau$_{181}$	Age	4.021	1.892	.211	2.126	.050
	Gender	-17.040	23.526	-.070	-.724	.628
	Family History	-3.763	25.727	-.014	-.146	.272
	BMI	-1.617	2.543	-.076	-.636	.520
	APOE ε4	-31.076	29.296	-.135	-1.061	.117
	HOMA-IR	-111.865	55.039	-.281	-2.032	.005*
	(APOE ε4)X(HOMA-IR)	218.175	77.190	.447	2.826	.017*
Regression Model Statistics						
R^2 = .120 F(7,105) = 1.888 *p*=.056						

Table 9. Summary of Multiple Regression Analysis for CSF P-Tau$_{181}$ (N = 113)						
Dependent Variable	Predictor Variable	B	SE(B)	β	*t*	Sig.(p)
P-Tau$_{181}$	Age	4.021	1.892	.211	2.126	.050
	Gender	-17.040	23.526	-.070	-.724	.628
	Family History	-3.763	25.727	-.014	-.146	.272
	BMI	-1.617	2.543	-.076	-.636	.520
	APOE ε4	-31.076	29.296	-.135	-1.061	.117
	HOMA-IR	-111.865	55.039	-.281	-2.032	.005*
	(APOE ε4)X(HOMA-IR)	218.175	77.190	.447	2.826	.017*
Regression Model Statistics						
R^2 = .120 F(7,105) = 1.888 *p*=.056						

Our secondary analysis of $A\beta_{42}$/P-Tau_{181} showed a significant interaction between *APOE* ε4 and HOMA-IR (β= -0.410, t(112)= -2.80 p= 0.006) on the $A\beta_{42}$/P-Tau_{181} ratio. Among *APOE* ε4 carriers only, there was a negative relationship between HOMA-IR and $A\beta_{42}$ /P-Tau_{181} as HOMA-IR increased, the ratio decreased.

We found no significant effects of family history on CSF biomarkers, nor were any significant interactions with family history observed.

FIGURE 5. INTERACTION BETWEEN INSULIN RESISTANCE AND *APOE* E4 PREDICTS P-TAU$_{181}$ AND TOTAL TAU



DISCUSSION

Higher IR in this sample of asymptomatic late-middle-aged adults was associated with increased T-Tau and P-Tau$_{181}$, and decreased Aβ_{142}/P-Tau$_{181}$ in *APOE* ε4 carriers. These results suggest a possible preclinical mechanism that may contribute to increased risk for AD related neurodegeneration among late middle-aged adults with glucoregulatory impairment.

Insulin resistance potentially promotes abnormal phosphorylation of tau in cognitively healthy middle-aged *APOE* ε4 carriers. Phosphorylation of tau decreases binding to microtubules and hyperphosphorylation eventually leads to formation of paired helical filaments aggregating to form neurofibrillary tangles. Impaired insulin signaling has been shown to enhance hyperphosphorylation of tau, which mediates dissociation of the protein from microtubules in the axons and association into tangles [96, 97]. IR has been implicated in tau hyperphosphorylation through upregulation of kinases, particularly downstream kinases that are known to phosphorylate tau protein, including glycogen synthase kinase 3β (GSK3β) [147, 148].

The relationship between type 2 diabetes, IR, and brain pathology in humans has been mixed, but may in part be explained by *APOE* ε4. In a postmortem study conducted by Malek-Ahmadi et al [149], AD patients who were diagnosed with type 2 diabetes and who were *APOE* ε4 carriers, showed higher plaque and tangle burden compared to non-carriers, suggesting that type 2 diabetes may exacerbate AD neuropathology in the presence of *APOE* ε4. This is consistent with the current findings, where *APOE* ε4 carriers showed greater CSF tau levels. Conversely, the general

prevalence of type 2 diabetes among AD patients is actually *higher* in *APOE* ε4 non-carriers compared to *APOE* ε4 carriers [150].

Underscoring the importance of *APOE* ε4 status specifically in relation to insulin, AD and MCI patients have shown differences in memory performance in response to intranasal insulin therapy based on *APOE* ε4 carriage. In one study, intranasal insulin therapy led to improved memory performance among *APOE* ε4 carriers [151], while in another study, facilitated memory function in *APOE* ε4 non-carriers [152]. Like intranasal insulin, intravenous insulin infusion shows differential effects among *APOE* ε4 carriers and non-carriers. Specifically, *APOE* ε4 non-carriers show memory facilitation in hyperinsulinemic conditions, an effect not observed among *APOE* ε4 carriers [21]. Basal levels of plasma and CSF insulin in AD patients also differ by genotype, with *APOE* ε4 homozygotes showing normal basal levels of insulin compared to heterozygotes and non-carriers [153], suggesting an importance of *APOE* ε4 load. Animal work suggests that insulin signaling may differ depending on *APOE* ε4 status. A study of *APOE* ε4 knock-in mice revealed altered insulin signaling when compared to *APOE* ε3 mice. Effector molecules including IRS-1, PI-3K and Akt phosphorylation were all decreased in the liver of *APOE* ε4 mice suggesting an *APOE* ε4 mediated alteration in insulin signaling [154]. More research is needed to determine whether the product of *APOE*, apolipoprotein E, is involved in molecular processes modulating the effect of insulin resistance on pathological markers of AD.

Interesting interactions have also been reported between insulin levels and cognitive status. Among non-demented older adults, higher serum insulin levels are associated with a deleterious effect on cognitive function in addition to greater global and hippocampal atrophy. [155] In contrast, among patients diagnosed with AD, higher insulin levels are associated with better cross-sectional and longitudinal outcomes, including less decline in cognitive function, and lower global and hippocampal atrophy [155, 156]. Taken together, these studies suggest the importance of considering genotype in treatments that target insulin signaling and the potential for non-linear relationships between IR and neural outcome across the spectrum of cognitive function. With the exception of an interaction between IR and *APOE* ε4 on $A\beta_{42}$/P-Tau_{181} ratio, we did not find a relationship between IR and CSF $A\beta_{42}$. Our group has previously found higher amyloid burden among normoglycemic WRAP participants with higher HOMA-IR, using Pittsburg compound B position emission tomography (PiB-PET) scanning [23]; although at least one prior study has found a negative relationship between IR and amyloid levels measured with PiB-PET [11]. Further work will be needed to determine the extent to which CSF levels of $A\beta_{42}$ correlate with regional amyloid deposition as shown with amyloid-PET in this preclinical population.

This study examined participants who were cognitively asymptomatic, and largely normal on fasting glucose and fasting insulin levels. Cut-offs for insulin resistance vary by study and the population under consideration, however, for comparison purposes, 85% of our sample fell below the cut-point of 3.8 for IR, while

75% of the sample was below the cut-point of 2.6 for IR [157-159]. Three participants were taking metformin for diabetes and one participant was identified who had elevated fasting glucose; however, removing these participants from the analyses did not change the relationship between IR and CSF markers of tau among *APOE* ε4 carriers, suggesting these relationships can be observed within the relatively normal variation of IR. While we have framed IR as key player in the generation of AD pathology we must note that the observed variation in IR itself could be the result of preclinical AD having an effect on metabolic regulation, for example, due to AD pathology in brain regions which regulate metabolic pathways [160]. Alternatively, AD pathology and peripheral insulin resistance could be co-occurring manifestations of either brain, or systemic metabolic dysfunction. This notion is supported by studies showing commonalities among diabetes, insulin resistance, and AD, including mitochondrial dysfunction [161-164]. The fact that these relationships can be detected in preclinical stages of glucoregulatory impairment however, does suggest the potential importance of preclinical detection and prevention strategies for mitigating age and disease associated brain changes in later life.

It is important to note that while CSF tau levels are known to associate with postmortem tangle burden [165], T-Tau is a non-specific marker and we cannot conclude that an AD-specific process is at play. The vascular effects of insulin resistance also cannot be discounted. Cerebrovascular pathology could lead to neural injury that in turn could lead to elevated levels of tau protein in CSF, particularly T-tau. Combined

CSF and blood flow brain imaging studies are expected to shed further light on vascular contributions to AD pathology, as well as provide a method for localizing pathology. Furthermore, although P-Tau is more specific to AD, P-Tau levels were only assessed for tau phosphorylated at site 181. There are over 80 sites for phosphorylation of tau and some of these sites may be more closely linked with insulin signaling disruption[147].

Other potential limitations of this study include the small sample size and the cohort's demographics, particularly in this sub-sample that underwent lumbar puncture. Participants were largely Caucasian, female, and had high levels of educational attainment, and thus are not representative of the general population. Finally, it is important to note that longitudinal investigation will be necessary to assess the long-term impacts of insulin resistance on disease progression.

Conclusion

This study demonstrates for the first time that pre-diabetic levels of IR are associated with elevated levels of CSF tau protein in an asymptomatic population with genetic risk for AD. This study suggests that insulin resistance may enhance tau pathology, especially in *APOE* ε4 carriers. Understanding modifiable factors during the preclinical stage is expected to contribute to treatment and prevention strategies in AD. The next study aimed to explore effect of midlife obesity on markers of amyloid and NFT pathology.

Study 4: Abdominal obesity and markers of amyloid and NFT pathology [Negative Findings]

Introduction:

Obesity at midlife is associated with a higher risk of developing dementia due to Alzheimer's disease (AD). Animal models suggest obesity may increase both tau and amyloid pathology[18, 166]. Body mass index is classically investigated as a measure of body habitus[167]. Given that visceral fat has different effects on metabolic processes compared to subcutaneous fat[168], our study focused on central obesity measurements as the circumference of the waist correlates with the fat that surrounds the organs. Mechanisms by which obesity affect the brain are yet to be clarified. Concerning AD specifically, animal studies suggest a significant correlation between obesity and amyloid pathology[20].

The purpose of this study was to determine whether abdominal obesity is associated with markers of neurofibrillary tangle and amyloid pathology measured in CSF of cognitively healthy middle-aged adults at increased risk for AD. We hypothesized that abdominal obesity would predict lower amyloid and higher tau. Further, given prior findings in our IR studies, we tested the extent to which obesity interacts with the ε4 allele of APOE gene to increase effects on AD pathology at midlife.

Methods

Participants.

Cognitively healthy participants (N=110, mean age= 60.5yrs, range=47-75yrs, 76 women) from the Wisconsin Registry for Alzheimer's Prevention study underwent lumbar puncture, blood draw, and measurement of waist circumference. The majority had a family history of AD (77%), and 36% were APOE4 carriers.

Lumbar Puncture and CSF Assay

Lumbar puncture was conducted according to the procedures described above, and CSF samples were assayed for markers related to AD pathology ($A\beta_{42}$, $A\beta_{40}$, T-tau, and P-Tau_{181})

Obesity and associated factors

Women were considered obese if waist circumference was greater than 88cm and men if they had a waist circumference larger than 102cm[169]. Hypertension and cholesterol index were determined by a baseline survey that asked if a participant has ever been diagnosed with hypertension or high cholesterol.

Statistical Analysis

The effects of central obesity on markers of AD pathology were analyzed using linear regression covarying for age, sex, hypertension index (1:hypertensive, 2:not hypertensive), cholesterol index (1:high cholesterol; 2: healthy cholesterol), APOE ε4 and log transformed insulin homeostatic model for insulin resistance (HOMA-IR) values.

Results:

We did not find significant effects of abdominal obesity on the CSF measures of AD biomarkers. Our model did not show waist circumference-determined obesity to be a direct significant factor in midlife AD pathology accumulation, or tau pathology. Variables that predicted CSF $A\beta_{42}$ pathology included APOE ε4 (β= -0.205, t(109)= -2.178, p= 0.032) genotype, and the hypertension index(β= 0.213, t(110)= 2.153, p= 0.034), as well as HOMA-IR which predicted CSF $A\beta_{40}$ (β= -0.316, t(109)= -2.852, p= 0.005).

Conclusion

Our results indicate abdominal obesity may not be the best predictor of amyloid or tau pathology. However, comorbidities of obesity, including insulin resistance and hypertension show significant associations with CSF biomarkers for amyloid pathology. Metabolic factors in the preclinical stages of AD may contribute to AD pathology. However, our results indicate abdominal obesity may not be more predictive of changes than overall body mass index. Perhaps the effects of obesity on AD pathology at midlife are mediated through another mechanism such as IR or vascular health[170, 171]. Another cohort study found that BMI significantly predicted increases in CSF AB and decreased in the cerebral amyloid deposition as measured by PIB-PET imaging[172], with the largest difference being the focus on BMI as opposed to visceral fat as considered in this study. Evidence for obesity at midlife contributing to decreased vascular health is explored further in **Appendix A**.

Interestingly, hypertension showed a positive relationship with AB in the CSF. Hypertension was considered as an ordinal variable based on prior diagnosis and self-report of this diagnosis. Self-report is a limitation. However, the results are intriguing. We also found a relationship between APOE4 and amyloid measures in the CSF, a finding that is consistent with prior studies[173]. Other limitations include the cross-sectional nature of this analyses, and that our measure of waist circumference is not the most accurate measure of visceral fat. Using a more specific measure such as computed tomography, MRI, or DEXA scanning would provide a more accurate measurement of visceral adiposity, and consequently a possibly better predictor of pathological consequences.

Summary

This study suggests abdominal obesity at midlife does not show marked effects on biomarkers for AD pathology[174], while showing some evidence for the adverse effects of hypertension and APOE4 genotype. It is of utmost importance that we understand the mechanisms of metabolic interference with AD development. Understanding the nuances of AD pathology difference and changes can help us provide better strategies for prevention and new therapeutic targets aimed at correcting the comorbidities of obesity.

Chapter Conclusion

In this chapter, we have explored the varying effects of insulin resistance, body mass index and abdominal obesity on biomarkers for amyloid and tau pathology in the CSF. Analyses show higher IR predicts decreased levels of amyloid biomarker and increases in NFT biomarkers but only in *APOE* ε4 carriers. Abdominal obesity does not seem to have any direct effect on CSF biomarkers for AD pathology yet co-morbid conditions such as hypertension and insulin resistance have significant associations with amyloid biomarkers. Analyzing CSF biomarkers about metabolic risk factors is a new area of study, and our analyses have provided some of the first evidence of the effects of metabolic factors on AD pathology in a cohort enriched for AD risk factors. A significant movement in the field of AD is to use biomarkers such as these to assess the probability of developing AD later in life. With that in mind understanding, modifiable factors during the preclinical stage are expected to contribute to prevention strategies and may even influence treatment.

Chapter 4: Insulin Resistance, obesity and Brain microstructure structure

Study 5: Insulin Resistance, waist circumference and white matter microstructure

Introduction

Brain atrophy in aging is a normal process and includes both gray and white matter loss [175]. In normal aging, the decline is gray matter volume is linear and begins around the third decade. In Alzheimer's disease (AD), there is greater atrophy than observed in healthy aging, and loss of neural cells and synapses corresponds with cognitive decline. Insulin resistance at midlife has been found to be related to the longitudinal decline in gray matter volume. Willette et al. previously observed that insulin resistance associated with both cross-sectional and longitudinally predicted gray matter atrophy in several brain regions including medial temporal lobe and portions of the hippocampus in cognitively healthy participants from the Wisconsin Registry for Alzheimer's Prevention (WRAP) study [176]. Studies comparing those with diabetes to healthy control also observed increased atrophy in the amygdala and hippocampi of diabetic participants while controlling for other vascular morbidities. While there is substantial evidence that diabetes and insulin resistance are related to gray matter changes less is known regarding the effect of insulin resistance on white matter.

White matter—mainly comprising axons and myelin—facilitates coordinated communication among different areas of the brain. Damage to white matter tracts alters the propagation of the signals, leading to slowed or decreased signal propagation and potentially manifesting as cognitive dysfunction. White matter microstructure can be assessed in vivo with diffusion tensor imaging (DTI). Measuring a signal related to water diffusion, DTI is sensitive to microstructural alterations that may not yet be

detectable by volumetric T1-weighted MRI. The two most widely reported measures generated from DTI include fractional anisotropy (FA), reflecting directional diffusion of water molecules, and mean diffusivity (MD), which is sensitive to the restriction of isotropic diffusion of water molecules. In aging and AD, as well as in several other conditions of pathology (particularly when cell loss is occurring), MD tends to increase, and FA is decreased compared to normal.

Several studies show alterations in white matter microstructure in AD patients compared to mild cognitive impairment and control[177-180]. The areas most affected by AD include gray and white matter in the medial temporal lobe, parietal lobe, and frontal lobe. Specific white matter bundles where microstructural differences appear in AD include the corpus callosum, cingulum bundle, superior longitudinal fasciculus, uncinate fasciculus and the fornix. Microstructural changes measured with DTI correlate with cognitive changes and mean diffusivity measured in the hippocampus is a strong predictor of progression to AD from mild cognitive impairment[181].

Interestingly, studies in diabetics have shown that cognitive performance correlates with white matter changes in subjects with type II Diabetes[182]. When comparing the waist-hip ratio to (BMI) to DTI measures, one group found an increase in white matter volume in the temporal lobes, parahippocampal gyrus, brainstem and cerebellum[183]. Another study showed that obese subjects versus non-obese had altered white matter microstructure[184]. However, there are still few studies that have examined the effect of IR and waist circumference on white matter microstructure in

middle-aged adults. Given the potential for both of these conditions to affect features of the neuron including the axon, in addition to contributing to myelin damage, the current study examined the effect of both insulin resistance and waist circumference on white matter health as assessed with DTI. I hypothesized that greater HOMA-IR and central obesity would be associated with lower FA.

Methods

Participants

Three hundred and three members of the WRAP cohort were used in this study. The average age was 59 years old at the time of imaging appointment. Please refer to Table 10 for more demographic traits.

HOMA-IR and Central Obesity

HOMA-IR and waist circumference were examined as a continuous variable to predict changes in fractional anisotropy. HOMA-IR was measured as previously described. Waist circumference was measured in centimeters. Measurements were taken from the region of the largest circumference.

DTI acquisition

Imaging was performed on a General Electric 3.0 T Discovery MR750 (Waukesha, WI) MRI system with an 8-channel head coil and parallel imaging with

ASSET. DTI was acquired using a diffusion-weighted, spin-echo, single-shot, echo planar imaging pulse sequence in 40 encoding directions, B0 = 1300, with eight non-diffusion weighted reference images. The cerebrum was covered using contiguous 2.5 mm thick axial slices, FOV = 24 cm, TR = 8000, E = 67.8, matrix = 96×96, resulting in isotropic 2.5 mm voxels. High order shimming was performed before the DTI acquisition to optimize the homogeneity of the magnetic field across the brain and to minimize EPI distortions. A T1-weighted volume was acquired in the axial plane with a 3D fast spoiled gradient-echo (3D EFGRE) sequence using the following parameters: TI = 450 ms; TR = 8.1 ms; TE = 3.2 ms; flip angle = 12°; acquisition matrix = 256×256×156, FOV = 260 mm; slice thickness = 1.0 mm.

Data were processed using a custom pipeline (as reported in Adluru et al., 2014). Head motion and image distortions due to eddy currents were corrected with affine transformation in the FMRIB Software Library (FSL) package (http://www.fmrib.ox.ac.uk/fsl/) The output changes from FSL eddy correction were used to reorient the diffusion gradients. Also, FSL was used to correct for geometric distortions introduced by the inhomogeneous magnetic field with a B0 field map and phase region expanding labeler for unwrapping discrete estimates (PRELUDE) and FMRIB's utility for geometrically unwarping EPIs (FUGUE). Brain tissue was extracted using FSL's Brain Extraction Tool (BET). Tensor fitting was performed using a nonlinear least squares method in CAMINO (http://cmic.cs.ucl.ac.uk/camino/).

Template creation

Registration of individual participant maps to a population-specific template was executed using Diffusion Tensor Imaging Toolkit (DTI-TK) (HTTP:// www.nitrc.org/projects/dtitk/) which is an optimized DTI spatial normalization and atlas construction tool [185-187] that has been shown to perform superior registration compared to scalar based registration methods [188]. The template is constructed to capture both the average diffusion and anatomical shape features in the population [186]. A population space template was generated from a subset of 77 diffusion tensor maps, from subjects that did not differ demographically from the primary sample. With DTI-TK, FA maps were calculated in the normalized space. All FA maps are visually inspected to rule out the inclusion of maps with missing data in regions of interest or other artifacts.

Spatial normalization.

White matter alignment was performed using a topology preserving registration method [189] that incrementally estimates the displacement field using a tensor-based registration formulation [187]. Tensor-based registration provides optimal alignment between subjects by taking advantage of similarity measures comparing whole tensors via explicit optimization of tensor reorientation [190, 191]. Image similarity is determined by full tensor images rather than scalar features allowing incorporation of local fiber orientation as features to drive the alignment of individual white matter

tracts. Using full-tensor information is highly effective in spatially normalizing tract morphology and tensor orientation, and enhances sensitivity to microstructural variations [189]. All diffusion tensor maps were normalized to the template with rigid, affine, and diffeomorphic alignments and interpolated to 2 × 2 × 2 mm voxels. With DTI-TK, FA maps were calculated in the normalized space, The order of processing steps was slightly different because diffusivities, but not fractional anisotropy, are more accurate when calculated in native space before warping.

Statistics

Voxel-wise whole brain analysis was conducted in SPM12. The predictor variables were HOMA-IR and central obesity. Thresholds were set at $p<.001_{uncorrected}$ and there was a minimum cluster threshold of 50 voxels. Thresholded results were saved, and figures were made in in MRIcron for display purposes.

Results

Demographics are shown in Table 10. The average age was 59 year of age, and the sample was predominantly female.

There was a significant effect of IR on FA. As shown in Figure 6, findings were mainly localized in bilateral internal capsule and bilateral posterior cingulum.

There was also a significant relationship between central obesity and FA. As shown in Figure 7, results were predominantly localized to the cerebellum and demonstrated a positive correlation between central obesity and FA ($p<.001_{uncorrected.}$).

Table 10. Participant Demographics N= 303	
	Mean ± S.D. (Range)
Age at MRI visit	59.2 ± 6.4 (42-73)
Sex (Female)	68.3% (207)
BMI (N=202)	28.27 ± 7
HOMA-IR	2.3 ± 2.3
Log of HOMA-IR	.24 ± .30
Diabetic	4.6%(14)
Pre-diabetic	20.1%(61)
Healthy	75.2%(228)
S.D. = Standard Deviation	

FIGURE 6. VOXEL-WISE ANALYSIS DISPLAYING AREAS OF INCREASED FA WITH INCREASING HOMA-IR

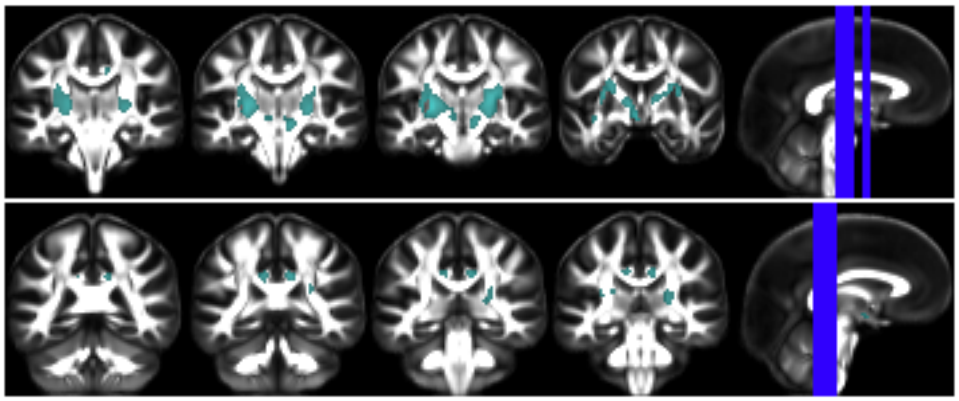
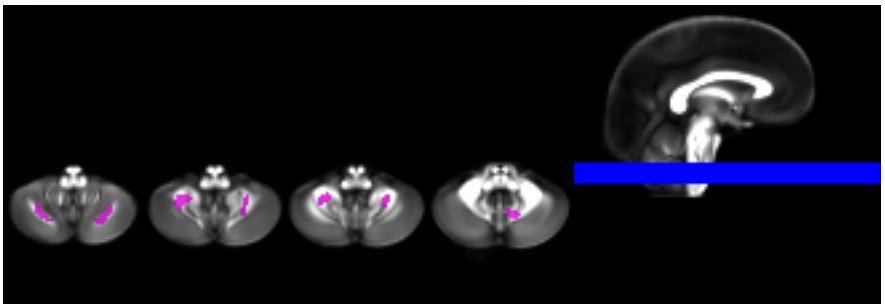


FIGURE 7. VOXEL-WISE ANALYSIS DISPLAYING AREAS OF INCREASED FA WITH INCREASING WAIST CIRCUMFERENCE



Discussion

This study examined the effects of insulin resistance and waist circumference on white matter microstructure in middle–aged cognitively healthy individuals. Waist circumference was associated with higher fractional anisotropy in the white matter of the cerebellum. HOMA-IR predicted higher fractional anisotropy bilaterally in the anterior and posterior internal capsule as well as the posterior cingulum.

Interestingly, the findings are in the opposite direction of what we hypothesized. Traditionally, decreases in anisotropy are considered a marker of neuropathology related to white matter disruption, including axonal degeneration or loss of myelin. More recently, however, some studies have reported increases in FA, although interpretations for these increases are sparse [192]. A predominating theory is that brain areas containing many crossing fibers may appear to have little FA on imaging, despite the microstructural complexity. Loss of crossing fibers due to disease or another adverse process could then lead to apparent increases in FA as it seems as if the anisotropy is increasing due to selective loss of fibers in one direction[193]. Another possibility is that neural injury in the cortex could lead to short-term increases in myelin repair processes, which manifest as higher FA. Interestingly, a recent multimodal imaging study assessing amyloid deposition with PiB-PET and white matter microstructure with DTI found higher white matter FA among individuals with greater Aβ deposition compared to those without significant PIB signal. Since we have previously observed insulin correlates with increased amyloid deposition, we could

speculate that IR leads to increased amyloid deposition which in turn could lead to increased compensatory mechanisms that affect white matter as previously reported by Racine [194].

We did not find any areas where IR or central obesity predicted decreased FA. Other studies have reported decreased FA in those with high HOMA-IR versus those with lower HOMA-IR. Ryu et al. examined FA and axial diffusivity in a middle aged sample and found reduced FA in the high HOMA-IR group compared to the lower HOMA-IR group[195]. This study differed in the age of the sample, as well as the cohort make-up, our sample being enriched for AD risk factors such as APOE4 genotype and parental family history. Adluru et al. have shown higher FA in the genu of the corpus callosum in participants from the WRAP study with a family history of AD[196]. Presumably, since our sample has a similar make-up of about 70% family history positive, this could affect our observations of fractional anisotropy, compared to Ryu et al. study.

We can only speculate as to the molecular processes at play underlying increased fractional anisotropy. Insulin resistance may be affecting myelin homeostasis. Insulin and insulin-like growth factor activate myelin basic protein, which is a fundamental component of the myelin sheaths made by oligodendrocyte glial cells[197, 198]. Insulin can act as growth factor and with insulin resistance, the accompanying hyperinsulinemia provides an opportunity for increased insulin signaling in the brain. Since these individuals are mostly metabolically healthy, the more detrimental

consequences of diabetes have not had a chance to develop fully. In other words, at the age of the participant, compensatory mechanisms may still be on working to maintain homeostasis in the face of abnormal insulin levels yet since there are increased insulin levels there may be some over signaling leading to increased activity. While the aforementioned processes are only speculation at this point, there are in-vivo and in-vitro models suggesting IR may have a role in myelin and axon structure and function.

At this point, we cannot make any conclusion about whether or not increase FA will predict conversion to AD in those with higher HOMA-IR. However, we can conclude that IR and waist circumference are having an effect on brain structure far before any clinical symptoms of diabetes or AD appear. Further investigation is needed to unravel the molecular processes involved in these brain changes, but I am hopeful as investigations are underway aimed at explaining the connection between metabolic dysfunction and AD.

Study 6: Interaction between APOE4 and IR predict changes in white and grey matter microstructure: A Diffusion Tensor imaging analysis

(Data are in preparation for publication)

Introduction

The prevalence of insulin resistance (IR) is increasing in the general population, and related conditions such as type 2 diabetes, metabolic syndrome, augment the risk of dementia due to Alzheimer's disease (AD)[94, 95, 199]. A growing body of evidence suggests IR may be involved in the pathophysiology of AD, amyloid plaques (AP), neurofibrillary tangles (NFTs), altered metabolism and hippocampal atrophy. For example, neuroimaging studies suggest an association with insulin resistance and augmented hippocampal atrophy, abnormal cognition [176], decreased cerebral glucose uptake [208], changes in white matter microstructure [201] and increases in amyloid plaques as measured by positron emission testing using Pittsburg Compound B (PET-PIB) [23, 105]. While IR has been found to alter white matter microstructure in middle-aged adults, less is know about the differences in the effect of IR between APOE ε4 carriers and non–carriers.

Understanding how APOE ε4 status and insulin resistance might interact in middle age to confer increased risk for development of AD will shed light on target mechanisms pharmacological modification. Currently, some studies suggest a differential effect of insulin resistance in APOE4 carriers versus non-carriers. While these findings are interesting, the relation to possible changes in neuronal structure is not well understood. Further, two studies assessing intranasal insulin treatment showed different effects in APOE ε4 status depending on the type of insulin administered (short acting vs. long-acting). For example, in a cross-sectional study

conducted by our group (Starks et al. 2015), we found a crossing interaction in a sample of asymptomatic late-middle-aged adults. In this interaction, APOE ε4 carriers demonstrated higher IR correlated with increased T-Tau and P-Tau181 and decreased Aβ142/P-Tau181[200].One idea is that loss of integrity of white matter leads to a disconnection of cognitive processes preceding decline into dementia.

Microtubules are highly significant to the cytoskeletal structure and integrity of the nervous system. Tau is a microtubule-associated protein involved in stabilizing microtubules. When tau becomes hyperphosphorylated, it no longer has a high affinity for the microtubules. NFTs are intracellular fibrillar structures composed of aggregated paired helical filaments made up of mostly hyperphosphorylated tau[211]. Neurofibrillary tangles deposit in a hierarchical, predictable pattern beginning in the transentorhinal cortex before spreading to the entorhinal cortex-amygdala, hippocampus and finally the neocortex [210]. Tau is important in maintaining the diameter of axons. Phosphorylated tau has a reduced affinity for microtubules, which could presumably lead to decreases in microtubule stability. In vitro analyses have demonstrated abnormal phosphorylation of tau to affect microtubule bundling [212] likely affecting axon structure and axonal transport processes. Given this, it is probable that decreases or increases in axonal diameter can affect the properties of water diffusion and would thus be measurable on diffusion tensor imaging (DTI).

DTI is an MRI method used for assessing microstructural changes by capitalizing on the Brownian motion of water molecules in the nervous systems. Movement of water

molecules provides information regarding the degree of isotropy and anisotropy in the white and gray matter[213-215]. From diffusion tensor MR imaging, it is possible to derive indices which reflect the diffusion characteristics (and, hence, the integrity and organization) of the tissue. One DTI index is mean diffusivity (MD) which is the measure of the average molecular motion independent of tissue directionality[215]. A second measure, fractional anisotropy (FA), reflects the degree of alignment, organization, and integrity of cellular structure within fiber tracts[216]. These DTI measures are sensitive to microstructural components such as axonal diameter, gliosis, myelin composition and fiber bundling. We aimed to utilize this neuroimaging to assess brain microstructure in middle age.

No study has examined the presence of an interaction between IR and APOE ε4 on the integrity of white and gray matter microstructure. Based on the prior knowledge that IR in APOE ε4 carriers shows differential effects on CSF tau, we proposed that IR in APOE ε4 carriers would associate with compromised white and gray matter microstructure integrity in brain regions known to be involved in Alzheimer's disease.

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Methods

Participants

Eighty-eight late-middle-aged adults enrolled in the Wisconsin Registry for Alzheimer's Prevention (WRAP), were assessed. Demographics are listed in Table 11. The WRAP study aims to understand how biological, medical and lifestyle factors affect

one's risk of developing AD. The cohort is mainly comprised of individuals enriched for AD with a family history of dementia due to AD and carriage of the APOE ε4 allele. All participants were required to have normal cognitive function, as determined by neuropsychological evaluation and consensus review, and no current diagnosis of major psychiatric illness.

Inclusion criteria for the present study required participants to have DTI scan data, cerebrospinal fluid assayed for AD biomarkers of AD and measures for fasting insulin and glucose. The sample had an average age of 60.3years old (SD = 5.8), 67% were female, 81% had a family history of dementia due to AD and 38% were carriers of at least one ε4 allele.

APOE ε4 extraction and isoform classification have been described previously [144]. Participants were categorized using a binary variable as being non-carriers (zero ε4 alleles) or APOE ε4 carriers (one or two ε4 alleles).

Homeostatic Model Assessment of Insulin Resistance and Body Mass Index

Fasting blood samples were collected following a 12hr fast during a clinical visit. Blood samples were processed at the University of Wisconsin Clinical Research Unit and fasting glucose, and insulin values were determined at the UW Hospital laboratory. Venous blood and CSF were collected the morning after a 12h fast. Blood samples were collected in 9ml polypropylene tubes, allowed to clot for 30mins and centrifuged at 4*C at 3000 rpm for 10mins. Cell-free plasma/serum was aliquoted into 1.5mL micro centrifuge tubes and frozen at -80 degrees Celsius. Plasma and serum samples were

analyzed at the University of Wisconsin Hospital and Clinics Hospital Laboratory (Madison, WI). To assess fasting glucose, plasma was assayed using glucose hexokinase method (Siemens Dimension Vista). To evaluate fasting insulin, serum was tested using chemiluminescent immunoassay on an ADVIA Centaur XP Immunoassay System (Siemens Corporation, Washington DC, USA). Participants' weight and height were measured without shoes. Weight was measured to the nearest tenth of a kilogram using a medical scale balance beam model, Health-O-Meter. Height was measured to the nearest centimeter using a wall-mounted ruler. Body mass index (BMI) was calculated based on height and weight.

Insulin resistance was indexed by the homeostatic model assessment of insulin resistance (HOMA-IR) calculated by the product of fasting glucose (mg/dL) and insulin (microunits per milliliter) divided by a constant, using fasting blood glucose and insulin from each participant. Fasting glucose and insulin values used to calculate HOMA-IR were taken from the visit nearest the time of imaging acquisition. Currently, cut-off points of HOMA-IR for insulin resistance are not well established. For example, studies in American Indians, American Mexican, Spanish, Japanese, and European populations demonstrate significant variability in HOMA-IR cut-offs and cut points have not been determined for a sample comparable to ours[217]. Thus, HOMA-IR was analyzed as a continuous variable. Because the raw data were skewed, all analyses used log-transformed (10) HOMA-IR. The presence of type 2 diabetes in this sample was assessed by reviewing medication records, self-report of diabetes diagnosis, and using

American Diabetes Association criteria, where participants with fasting blood glucose over 125 mg/dL were flagged as diabetic. Four participants had previously been diagnosed with type 2 diabetes and were taking Metformin. One participant not diagnosed with diabetes had elevated fasting glucose according to ADA criteria. Medications were also reviewed to identify additional participants with diabetes. To test for relationships in the prediabetic range, the analyses were repeated excluding participants with diagnosed or possible diabetes. The results did not differ. Thus, these 4 participants were retained.

Cerebrospinal fluid processing

CSF was collected in the morning after a 12hr fast with a Sprotte 25-or 24-gauge spinal needle at the L3/4 or L4/5 using gentle extraction into propylene syringes. CSF (~22mL) was then combined, gently mixed and centrifuged at 2000g for 10 minutes. Supernatants were frozen in 0.5mL aliquots in polypropylene tubes and stored at -80oC. Samples were analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) methods (INNOTEST assays, Fujiurebio, Ghent Belgium) as described previously in detail [145]. Board-certified laboratory technicians who were blinded to clinical information performed all analyses. All samples were analyzed according to protocols approved by the Swedish Board of Accreditation and Conformity Assessment (SWEDAC) using one batch of reagents (intra-assay coefficients of variation <10%).

Magnetic Resonance Imaging Acquisition and Analysis.

DTI acquisition

Imaging was performed on a General Electric 3.0 T Discovery MR750 (Waukesha, WI) MRI system with an 8-channel head coil and parallel imaging with ASSET. DTI was acquired using a diffusion-weighted, spin-echo, single-shot, echo planar imaging pulse sequence in 40 encoding directions, B0 = 1300, with eight non-diffusion weighted reference images. The cerebrum was covered using contiguous 2.5 mm thick axial slices, FOV = 24 cm, TR = 8000, E = 67.8, matrix = 96×96, resulting in isotropic 2.5 mm voxels. High order shimming was performed prior to the DTI acquisition to optimize the homogeneity of the magnetic field across the brain and to minimize EPI distortions. A T1-weighted volume was acquired in the axial plane with a 3D fast spoiled gradient-echo (3D EFGRE) sequence using the following parameters: TI = 450 ms; TR = 8.1 ms; TE = 3.2 ms; flip angle = 12°; acquisition matrix = 256×256×156, FOV = 260 mm; slice thickness = 1.0 mm.

Data were processed using a custom pipeline (as reported in Adluru et al, 2014). Head motion and image distortions due to eddy currents were corrected with affine transformation in the FMRIB Software Library (FSL) package (http://www.fmrib.ox.ac.uk/fsl/) The output transformations from FSL eddy correction were used to reorient the diffusion gradients. In addition, FSL was used to correct for geometric distortions introduced by the inhomogeneous magnetic field with a B0 field map and phase region expanding labeler for unwrapping discrete

estimates (PRELUDE) and FMRIB's utility for geometrically unwarping EPIs (FUGUE). Brain tissue was extracted using FSL's Brain Extraction Tool (BET). Tensor fitting was performed using a nonlinear least squares method in CAMINO (http://cmic.cs.ucl.ac.uk/camino/).

Template creation

Registration of individual participant maps to a population specific template was executed using Diffusion Tensor Imaging Toolkit (DTI-TK) (http:// www.nitrc.org/projects/dtitk/) which is an optimized DTI spatial normalization and atlas construction tool [185-187] that has been shown to perform superior registration compared to scalar based registration methods [188]. The template is constructed to capture both the average diffusion and anatomical shape features in the population [186]. A population space template was generated from a subset of 77 diffusion tensor maps, from subjects that did not differ demographically from the main sample. With DTI-TK, FA maps were calculated in the normalized space. All FA maps are visually inspected to rule out inclusion of maps with missing data in regions of interest or other artifacts.

Spatial normalization.

White matter alignment was performed using a topology preserving registration method [189] that incrementally estimates the displacement field using a tensor-based

registration formulation [187]. Tensor-based registration provides optimal alignment between subjects by taking advantage of similarity measures comparing whole tensors via explicit optimization of tensor reorientation [190, 191]. Image similarity is determined on the basis of full tensor images rather than scalar features allowing incorporation of local fiber orientation as features to drive the alignment of individual white matter tracts. Using full-tensor information is highly effective in spatially normalizing tract morphology and tensor orientation, and enhances sensitivity to microstructural variations [189]. All diffusion tensor maps were normalized to the template with rigid, affine, and dieffeomorphic alignments and interpolated to 2 × 2 × 2 mm voxels. With DTI-TK, FA maps were calculated in the normalized space, The order of processing steps was slightly different because diffusivities, but not fractional anisotropy, are more accurate when calculated in native space prior to warping.

Statistics

Multiple Linear Regression analyses were conducted in SPM12 (REF) on DTI measurements were APOE ε4, IR, and the interaction between APOE ε4 and IR were the predictor variables. Separate linear regressions were performed with FA and MD maps as the dependent variable. Mean- centered age, sex, BMI and family history (FH) of dementia due to AD were included as covariates because of the known or potential effects they might have on brain structure.

Exploratory whole-brain analyses were performed to test for the interaction between IR and APOE ε4 on FA and MD. Analyses were masked using white matter and gray matter masks generated using Atropos, an ITK-based multivariate n-class open source segmentation algorithm distributed with Advanced Normalization Tools or ANTs (http://www.picsl.upenn.edu/ANTs) [218]. To increase the anatomic plausibility of our results, we used a cluster extent threshold of N=50 contiguous voxels and an initial voxel-level threshold of $\alpha = 0.001$.

In order to determine the association between FA values generated with DTI and CSF biomarkers, raw values were extracted from regions of interest using the MarsBar toolbox implemented in SPM12. Derived values were adjusted in SPSS for age, sex and family history. A multiple regression analysis was conducted using SPSS (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) software to examine the relationship between FA and levels of CSF proteins T-tau and P-tau181. Separate analyses were performed for each CSF measure of interest.

Axial diffusivity (AD) and radial diffusivity (RD) are directional components of the diffusion tensor which are more accurate to biological correlates of diffusion changes. RD is reflective of changed in myelin and axonal diameter while AD is more representative of the long axis of the axon. A secondary analysis of AD and RD was performed using a mask generated from the significant clusters from the main analysis and small volume correction. The mask was created in SPM by first saving the

thresholded FA map then creating a binary image to be used for the region of interest analysis.

Finally, threshold SPM t-maps were saved and were registered to MNI space using FSL FLIRT for viewing and analysis purposes (3. M. Jenkinson, C.F. Beckmann, T.E. Behrens, M.W. Woolrich, S.M. Smith. FSL. NeuroImage, 62:782-90, 2012).

Results

Sample Characteristics

Demographics and clinical characteristics of the participants are listed in Table 1. Mean MMSE across all participants was within the normal range (29.38 ± .79). Average HOMA-IR was 2.2 (SD = 2.1), with untransformed values ranging from 0.45-7.65. A one-way ANOVA was conducted to test for significant differences between *APOE* ε4 carriers (n=34) and non-carriers (n = 54) on demographic and baseline metabolic variables (Table 1). No significant differences were observed between *APOE* ε4 carriers and non-carriers for age, sex, FH, MMSE score, BMI, HOMA-IR and average CSF P-tau and T-tau concentrations. Diastolic blood pressure bordered on being significantly higher among *APOE* ε4 carriers (p=.051) however, the significance of this difference is currently unknown. Overall our sample contained a significant proportion of *APOE* ε4 carriers, and when comparing carriers and non-carriers, no significant differences were observed.

Table 11. Participant demographics				
		APOE ε4		
	All	Carrier (38%)	Non-carriers (62%)	*p*
N	88	34	54	
Age (years)	60.3 ± 5.8 (47-71)	60.4 ± 6.2	60.4 ± 5.8	.923
Sex (Female)	67% (59)	73%(25)	62% (34)	.214#
Mini mental examination	29.39 ± .79	29.56 ± .74	29.28 ± .81	.106
Family History Positive	81%(71)	82%(28)	79%(43)	.490#
BMI	28.1 ± 4.7 (18.9-41.5)	27.9 ± 5.2	28.2 ± 4.4	.771
Fasting plasma Glucose	95.3 ± 10.1 (74-126)	94.2 ± 10.9	96.0 ± 9.6	.401
Fasting Plasma Insulin	9.33 ± 7.5 (2-53)	9.88 ± 9.5	8.8 ± 6.2	.516
HOMA-IR	2.2 ± 2.1	2.3 ± 1.69	2.2 ± 1.6	.629
Log of HOMA-IR	.24 ± .28 (-.35-1.2)	.25 ± .30	.23 ± .28	.791
Cerebrospinal Fluid (pg/mL)				
P-Tau$_{181}$	42.8 ± 14.1	42.1 ± 14.7	43.3 ± 13.8	.695
T-Tau	314.9 ± 108.5	323.0±105.7	309.74 ± 111.0	.579
Cardiovascular Risk factors				
Blood pressure mmHg				
Systolic	125.62 ± 15.94	123.00 ± 2.13	127.28 ± 17.71	.222
Diastolic	74.55 ± 9.63	72.03 ± 9.30	76.13 ± 9.58	.051
Total Cholesterol	198.545 ± 36.90	199.12 ± 34.76	198.19 ± 38.50	.909
# Chi-square				

Voxel-wise DTI analysis

An interaction between *APOE* ε4 and HOMA-IR was observed revealing that *APOE* ε4 carriage and higher IR was associated with significant increases in FA in the right medial temporal lobe, left insula, and areas of the occipital and parietal lobes. For APOE ε4 non-carriers there was a negative association in these regions (Figure 8, Table 12). A significant *APOE* ε4 x HOMA-IR interaction was also observed with MD were *APOE* ε4 carriage, and higher HOMA-IR was associated with higher MD in the right corona radiata ($p < .001_{\text{uncorrected}}$).

Fractional anisotropy is related to axonal health. Tau is also related to axonal health. While FA is very sensitive to microstructural abnormalities, it does not provide precise information regarding the *underlying* physiological etiology. Radial diffusivity(RD) and axial diffusivity(AD) provide more specific information about the type of physiologic insult leading to an altered FA measurement.

To further investigate the increases in FA, two post hoc analyses were conducted to understand the physiologic relevance of the interaction using CSF biomarkers and DTI measures of AD and RD.

FIGURE 8. T-MAP OF THE SIGNIFICANT CLUSTERS IN APOE4 AND INSULIN RESISTANCE INTERACTION.

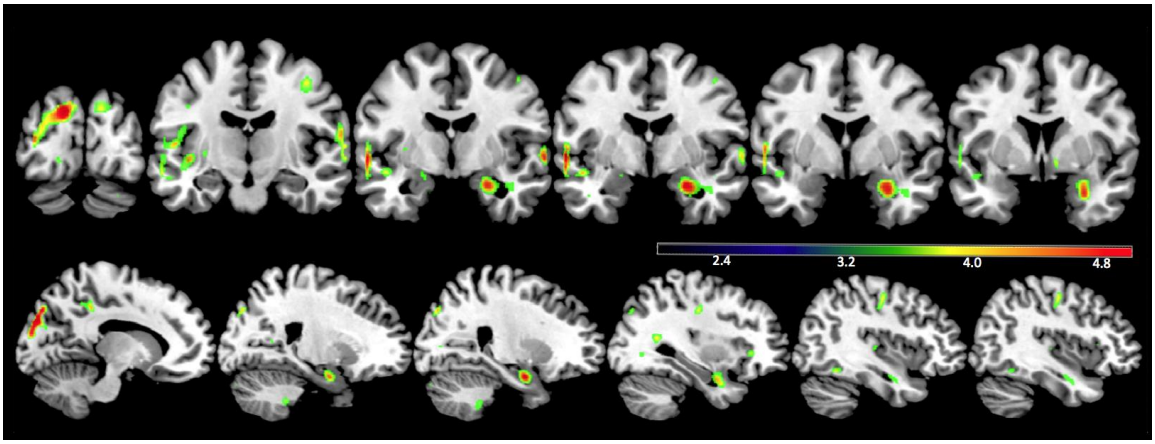

Table 12. Neuroanatomical regions of significant clusters with increase FA in APOE4 carriers with higher HOMAIR. P<.001

	MNI coordinates	Peak t-value	K(mm^3)
WM Left Cuneus	-11 -87 28	5.35	528
	-10 -84 36	5.09	
Broadmann18	-13 -90 19	4.94	
WM R superior temporal gyrus	30 7 -28	5.08	162
Amygdala	27 -2 -22	5.05	
Left superior Temporal Lobe (broadmann21)	-57 -3 -3	4.88	180
WM Left middle temporal lobe	-60 -17 -12	4.35	
WM Right postcentral gyrus	63 -14 16	4.77	348
Right superior temporal gyrus	64 -5 -1	4.75	
R Middle temporal gyrus	67 -8 -3	4.28	
WM Left Superior temporal lobe	-47 8 -18	4.39	129
	-52 14 -13	4.27	
	-41 12 -3	4.25	
WM Right Precentral Gyrus Frontal lobe	35 -17 37	4.32	70
	41 -13 48	3.90	
Right precuneus Broadmann 19	25 -80 38	4.21	105
WM Right cuneus	14 -86 32	4.00	
WM Right precuneus	33 -76 38	3.75	

***APOE* ε4, HOMA-IR interaction and radial diffusivity, axial diffusivity**

Using SPM, we generated a mask of the significant areas from the *APOE* ε4, HOMA-IR interaction analysis. Meaning, any clusters that were significant in the previous analysis, we assessed for changes in RD and AD. We chose a more lenient threshold of $p<.005_{uncorrected}$, due to the small volume analysis. Upon analysis, one cluster in the amygdala was significant for changes in AD and RD. In APOE ε4 carriers, AD increased, and RD decreased with increased HOMA-IR. The opposite was true for *APOE* ε4 non-carriers, AD decreased, and RD increased with increasing HOMA-IR in the amygdala. In the amygdala, we see *APOE* ε4 carriers show increased FA and RD with decreased AD while non-carriers show reduced FA and AD with increased RD. These DTI measure profiles could be related to myelin or axonal changes. Tau is a major component of axon structure so we aimed to assess the relationship between *tau* 4and DTI measures, with the idea that CSF tau might aid in interpreting the increases in FA seen in the APOE ε4 carriers.

FIGURE 8 .VALUES EXTRACTED FROM THE RIGHT AMYGDALA PLOTTED AGAINST HOMA-IR.

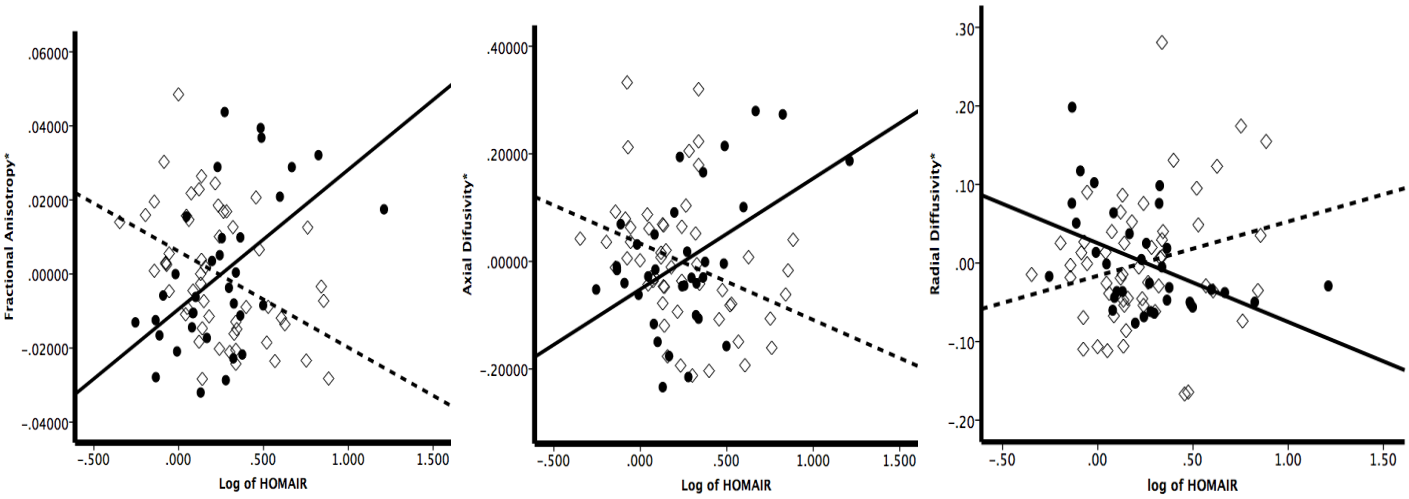

Table 13. Significant relationship between FA measures and CSF tau

	P-tau		T-tau	
	beta	p-value	beta	p-value
WM Left Cuneus	.268	.011	.099	.345
WM R medial temporal gyrus	.255	.017	.277	.008
Right Amygdala	.316	.002	.303	.003
Left superior Temporal Lobe (broadmann21)	.189	.080	.105	.317
Right superior temporal gyrus	.229	.032	.145	.165
WM Left middle temporal lobe	.266	.011	.229	.025
WM Right Precentral Gyrus Frontal lobe	.144	.185	.154	.144
WM Right precuneus	.118	.281	.010	.925
	Based on peak voxel of the cluster			

Cerebrospinal Fluid Markers and DTI measures

Using CSF measures closest to the MRI date, we performed linear regression analyses in SPSS to assess the relationship between the FA values and markers of axon health phosphorylated tau (P-tau) and total tau (T-tau). To do this, mean FA was extracted from the medial temporal lobe using the MarsBaR toolbox in SPM12 and analyses were adjusted for age, sex, BMI and family history of dementia due to AD. Our model included FA peak voxel values from clusters including the left cuneus, right and left superior temporal lobe, amygdala, the left medial temporal lobe, right precentral white matter, and the right precuneus (Table 13). Covariates included age, sex, BMI and FH, and white matter hyper intensities.

Significant relationships between FA values and P-tau$_{181}$ included the left cuneus (β= 0.268, t(87)= 2.589 p= 0.011), right medial temporal lobe (β= 0.255, t(87)= 2.428 p= 0.017), the right amygdala (β= 0.316, t(87)= 3.154 p= 0.002), right superior temporal lobe (β= 0.229, t(87)= 2.176 p= 0.032) and left middle temporal lobe (β= 0.266, t(87)= 2.588 p= 0.011).

Significant relationship between extracted FA values and T-tau were observed from clusters that included right superior temporal lobe (β= 0.277, t(87)= 2.739 p= 0.008), amygdala (β= 0.303, t(87)= 3.118 p= 0.003) and left medial temporal lobe (β= 0.229, t(87)= 2.279 p= 0.025). One participant was observed to be an outlier for CSF P-Tau, removal of this participant a re-analysis left a significant effect in the right

amygdala and left medial temporal lobe while the other relationships with P-tau become trending. Graphs of the particular relationships and area they are drawn from are displayed in figure 1.

To further query the significant interaction between HOMA-IR and APOE4 on MD, a linear regression was performed where right corona radiata MD was the independent variable, and the cerebrospinal fluid markers were dependent variables. There were no significant associations between MD and any of the cerebrospinal fluid markers. This finding makes us more confident of a relationship between tau and FA.

Discussion

Consistent with prior findings, the results of the current study provide further evidence that the effect of IR on brain health differs by *APOE* genotype. Further, our results suggest differing brain microstructure in *APOE* ε4 carriers versus non-carriers. Invariable risk factors such as *APOE* ε4 carriage may interact with modifiable risk factors such as insulin resistance at midlife, and prior studies suggest that the effects of IR may differ depending on APOE4 genotype.

While we expected that higher IR among *APOE* ε4 carriers would be associated with lower FA, we found that higher IR among *APOE* ε4 carriers was paradoxically associated with increased FA, while *APOE* ε4 non-carriers showed decreases in FA with higher IR. Notably, the areas affected included the white matter of the right frontal lobe (precentral gyrus), right parietal lobe (postcentral gyrus), white matter of the left and

right cuneus, the left precuneus and white matter of the left and white superior temporal lobe and finally the amygdala.

These results could be interpreted in different ways. Because lower FA is often construed as reflecting a greater underlying pathological process, the results might suggest that APOE4 carriage is somehow protective in the presence of higher IR, while the opposite is true in APOE4 non-carriers, which may show greater effects of abnormal insulin signaling. To further understand the paradoxical finding in FA, a secondary analysis examined maps that are more specific to myelin and axonal alteration, RD and AD respectively. Among areas of increased FA, correspondingly higher axial diffusivity, and lower radial diffusivity were observed for APOE4 carriers with higher IR. Interestingly, a motivating prior study from our group suggests that APOE4 carriers with higher IR have higher CSF P-tau181 and t-tau (Starks et al. 2015). One possibility is that the higher tau measures in CSF, coupled with the altered microstructure on DTI suggest greater tau turnover among the APOE4 carriers, although it is not possible to firmly make this conclusion from indirect measures. Overall, the findings do suggest that the effects of IR on brain microstructure depend on *APOε4* status.

APOE ε4 and Insulin resistance

These results are consistent with literature reporting differential effects of IR between *APOE* ε4 carriers and non-carriers. Increasing evidence suggests IR has a role in AD pathophysiology, and targeting insulin dysregulation offers a viable treatment

option for AD. Interestingly some studies have established that both the deleterious effects of IR and the treatments targeting may be moderated by the carriage of the APOE ε4 allele[4, 24]. In one study of patients with memory impairment, intranasal insulin therapy led to improved total story recall only among *APOE* ε4 non-carriers [25]. In another study, the insulin sensitizer rosiglitazone facilitated verbal recall only among *APOE* ε4 carriers [26]. Comparable to intranasal insulin, intravenous insulin infusion shows differential effects among *APOE* ε4 carriers versus non-carriers. Notably, *APOE* ε4 non-carriers show memory facilitation in hyperinsulinemic conditions, an effect not observed among *APOE* ε4 carriers [27]. Basal levels of plasma and CSF insulin in AD patients also differ by genotype, with *APOE* ε4 homozygotes showing normal basal levels of insulin compared to heterozygotes and non-carriers [28], suggesting an importance of *APOE* ε4 load. Importantly, CSF tau levels are known to associate with tangle postmortem burden [29].

Animal work suggests that insulin signaling may differ depending on *APOE* ε4 status. A study of *APOE* ε4 knock-in mice revealed altered insulin signaling when compared to *APOE* ε3 mice. Effector molecules including IRS-1, PI-3K, and Akt phosphorylation were all decreased in the liver of *APOE* ε4 mice suggesting an *APOE* ε4 mediated alteration in insulin signaling [30]. All of the previous evidence provides support for the theory suggesting insulin aberrations may be more detrimental for *APOE ε4* non-carriers. Further, our data suggests insulin resistance in apoe4 non–

carriers could potentially elicit early microstructural changes. In the next section, we will explore the meaning of these different microstructural profiles.

Diffusion Tensor Imaging

These results indicate the presence of a significant interaction between *APOE* ε4 and IR. Specifically, *APOE* ε4 carriers with higher IR showed greater FA and *APOE* ε4 *non*-carriers with higher IR showed lower FA. Neuropathologic correlates of higher FA in humans are poorly understood, while decreases in FA could be associated with altered axons or myelin[31, 32]. Several DTI studies in mouse models of AD have found increased FA in regions, even some that are considered grey matter, including the cingulate, striatum, thalamus, hippocampus, corpus callosum, anterior commissure, internal capsule, septal nuclei, caudate, putamen, fimbria, right piriform cortex, hypothalamus, and external capsule [33, 34] [35, 36]. Abnormal maturation of fibers, myelin degeneration in high fiber brain structures, complex nuclei structure, loss of isotropic cells, decreased the proportion of unmyelinated fibers, and loss of crossing fibers are all possible biological explanations for apparent increases in microstructural anisotropy[37]. While animal studies provide a framework for interpretation of the data, human studies have not fully explored the underlying physiology of increased fractional anisotropy.

Some studies have reported increases in FA yet failed to completely understand its significance. To further interpret the results of higher FA in our study, significant

clusters were further investigated in each region using radial diffusivity and axial diffusivity maps. In *APOE* ε4 carriers with higher IR, there was a pattern of increased FA, decreased D_r and increased D_a. The reverse pattern of decreased FA, increased D_r and decreased D_a was observed among the *APOE* ε4 non-carriers with higher IR. Significant D_r, and D_a effects were only found in the right amygdala. Increases in D_a suggest a loss of crossing fibers or could relate to the increases in myelination but more likely has to do with axon structure [38, 39]. Decreases in D_r may correspond with areas of high myelination, dense axonal packing and white matter maturation[40]. One potential explanation as to why there would be increased myelination is due to abnormal insulin signaling and could also be related to the cholesterol-carrying properties of *APOE* ε4. *APOE* ε4 non-carriers show a more traditional pathological pattern suggestive of disruptions in axons and decreased myelin content. It seems midlife IR may confer a worse prognosis for brain health in those who are not carriers of an *APOE* ε4 allele.

Interestingly, we found significant differences in FA among gray matter regions, specifically the amygdala. Anisotropy in unmyelinated areas is supported by research showing measurable FA in unmyelinated neonate rat brains [31], likely due to the organized nature of axons. Other structures that exhibit anisotropy, although at a much smaller magnitude than white matter, include the thalamus and a region where we found significant effects, the amygdala [41, 42].

In our study, of particular interest were the findings of increased anisotropy in the amygdala. The amygdala is a gray matter structure in the dorsomedial portion of the medial temporal lobe important for regulation of emotion, specifically the fear response, motivation, memory and learning [43, 44]. Among patients with AD, there are significant NFT deposits in the amygdala compared to non-demented controls [45]. FA measured in the amygdala has previously been shown to be related to amygdala microstructure [41]. Although there is little myelination as observed in postmortem cross-sections of the amygdala, there is a high density of fibers. According to Braak staging, the amygdala is one of the earliest sites of NFT deposition, suggesting that the tau measures from CSF observed in our study could reflect tau alterations in the axons of the amygdala, and possibly that axonal dysregulation may be the predominate biological mechanism at play.

We can only speculate about the molecular interactions potentially involved, as they are not well characterized in humans however animal studies offer some valuable clues as to the impact of insulin resistance on AD pathology. Disruption in insulin signaling is associated with increased phosphorylated tau in animal models as a result of decreased regulation of glycogen synthetase kinase-3 (GSK-3). Hyper phosphorylation of tau about insulin resistance is postulated to result from deregulation of kinases and phosphatases in the neuron[46], including glycogen synthetase kinase-3 (GSK-3), an enzyme essential for the regulation of metabolism through glucose storage and necessary for neurodevelopment [47]. GSK-3 is a predominant kinase in tau

phosphorylation [48, 49]. GSK-3 is inhibited by insulin signaling, However, in insulin resistance the downstream signaling is affected, and GSK-3 is not inhibited leaving it to phosphorylate substrate in the neuron [50].

Alternatively, the microtubule cytoskeleton is a dynamic structure necessary for transporting materials down the axon for proper neuronal signaling[51]. Normally there is a balance between p-tau and hypophosphorylated tau. Increased tau phosphorylation could tip the balance of tau and p-tau chemical equation, leading to increased synthesis of tau to compensate for the increased levels of tau phosphorylation. Increases in this protein could manifest in increased axonal integrity while the excess is excreted, depositing in the CSF.

Overall the findings suggest that higher IR in mid-life may increase the risk for loss of microstructural structural integrity of brain regions known to play a vital role in memory function and that *APOE* ε4 non-carriers may be at greater risk for pathologic alterations in brain areas associated with AD, especially the medial temporal lobe.

Limitations

It is important to note that while CSF tau levels are known to associate with tangle postmortem burden [29], T-tau is a non-specific marker, and we cannot conclude that an AD-specific process is at play. The vascular effects of insulin resistance also cannot be discounted. Cerebrovascular pathology could lead to neural injury that in turn could result in elevated levels of tau protein in CSF, particularly T-tau. Combined

CSF and brain imaging studies are expected to shed further light on vascular contributions to AD pathology, as well as provide a method for localizing pathology. Furthermore, although P-tau is more specific to AD, P-tau levels were only assessed for tau phosphorylated at site 181. There are over 80 sites for phosphorylation of tau, and some of these locations may be more closely linked with insulin signaling disruption[52].

Other potential limitations of this study include the limited sample size and the cohort's demographics, particularly in this sub-sample that underwent lumbar puncture. Participants were mainly Caucasian, primarily women, and had high levels of educational attainment, and thus are not representative of the general population. Finally, it is important to note that longitudinal investigation will be necessary to assess the long-term impacts of insulin resistance on disease progression.

Conclusion

Insulin resistance represents an important modifiable risk factor for AD. The current study provides evidence that in cognitively healthy late-middle-aged individuals with prediabetic levels of IR show changes in brain microstructure associated with early deposition of AD pathology. More specifically *APOE* ε4 non-carriers demonstrated a more pathologic profile which is in concordance with insulin therapy studies showing an increased benefit of insulin for non-carriers. This study provides further support that insulin resistance may interact with *APOE* ε4 alleles

differently at the molecular level in humans. These results add to a growing body of evidence suggesting that abnormalities to insulin signaling may contribute to the pathophysiology of AD. Understanding modifiable risk factors, especially those at mid-life are expected to contribute to the prevention and treatment of AD.

Conclusion

The preceding studies were aimed at understanding the possible mechanistic links between mild-life risk for AD and diabetes and obesity. Our studies suggest an upregulation of pathological processes in midlife with increasing insulin resistance. Higher levels of insulin resistance predicted lower levels of amyloid biomarkers in the CSF indicative of decreased clearance of Aβ peptide from the brain parenchyma. Further, our studies suggest overall body habitus, as measured by BMI, positively correlates with increases in CSF amyloid biomarkers, indicative of more effective clearance mechanisms. However when using abdominal obesity as determined by waist circumference as the predictor variable we failed to observe a significant relationship. However our model indicated that medical history of hypertension was associated with amyloid biomarkers indicating consequences of abdominal obesity may affect brain health predominantly via vascular mechanisms. Further, insulin resistance correlated with increases in phosphorylated tau and total tau in the CSF of APOE ε4 carriers but showed decreases in APOE ε4 non-carriers. In addition, we found an association with white matter and grey microstructure with increasing insulin

resistance; suggesting molecular processes are also affecting the cellular processes in midlife. Finally, we observed a longitudinal association between insulin resistance and increased rates of decline in processing speed and BMI with increased rates of decline in executive function.

While this research provides important insights into the mechanisms of IR and obesity at midlife, we can only speculate as to molecular mechanisms governing the effects we see. Novel techniques are arising that can help more clearly establish an understanding of in-vivo consequences of early deposition of AD pathology. Future studies should aim to use new imaging techniques such as tau and amyloid imaging in combination with cerebrospinal fluid measures of insulin and molecular imprints affected by obesity.

Understanding these connections between midlife metabolic risk factors can aid in the development of novel therapies centered on correcting metabolism that can slow or perhaps prevent the development of AD in those who are at an increased risk due to un-modifiable risk factors.

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Appendix A

Insulin resistance and cognition: Cross-sectional findings from the Wisconsin Registry for Alzheimer's Prevention

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ABSTRACT

Background: Type 2 diabetes increases the risk of Alzheimer's disease (AD) and both clinical and pre-clinical hypergylcemia are characterized by insulin resistance. Midlife appears to be a particularly vulnerable life-stage where cardiovascular risk factors are predictive of later cognitive decline. The current study assessed the relationship between insulin resistance, obesity, and cognitive function in a non-demented middle-aged cohort, the Wisconsin Registry for Alzheimer's Prevention. Primary analyses centered around four questions: 1) What is the relationship between insulin resistance and cognition? 2) How does adding obesity to the model affect that relationship? 3) Does gender interact with either of these metabolic predictors in affecting cognition? And 4) Do risk factors for Alzheimer's (family history or APOE4 status) interact with either metabolic predictor in influencing cognition?

Methods: Data used for this analysis were from the Wisconsin Registry for Alzheimer's Prevention study, utilizing data from the first visit where fasting labs were available (Wave 2, N= 975). In addition to fasting labs, participants underwent comprehensive cognitive testing. Factor analysis was used to reduce the set of cognitive measures to five factors: two factors represented verbal learning and recall (Immediate Memory and Verbal Learning and Memory), and two factors reflected components of executive function (Working Memory and Speed and Flexibility), in addition to a Visuospatial Memory factor. HOMA-IR (insulin × glucose)/405 was used as a measure of insulin resistance. Central obesity was defined based on waist measurements. Analyses were conducted using SAS (version 9.3). Linear mixed models were used to examine relationships between cognition, metabolic predictors, sex, and familial/genetic risk.

Results: We found a significant negative effect of HOMA-IR on Speed & Flexibility (β = -0.065, $F(1,964) = 5.09$, $p = .024$). Interestingly, when participants

with Type 2 diabetes were excluded, no effects of HOMA-IR were significant ($p > .05$). There was no significant main effect of obesity on cognitive performance ($p > .10$). When testing for interactions with AD risk factors, we found a significant APOE by HOMA-IR interaction ($F(1,944) = 7.00$, $p = .0083$) and a marginal FH by HOMA-IR interaction ($F(1,955) = 2.92$, $p = .088$). To explore simple main effects, separate mixed models were run for ε4- and ε4+ groups, and these suggested that the effect of HOMA-IR was significant for ε4- participants only (ε4-: $\beta = -0.11$, $F(1,586) = 6.55$, $p = .011$; ε4+: $\beta = .0074$, $F(1,361) = 0.02$, $p = .89$). Central obesity did not interact with AD risk factors.

Conclusion: Taken together, the results suggest that insulin resistance is associated with cognitive function only when participants with clinically diagnosed glucoregulatory dysfunction are included in the sample. Furthermore, the results indicate that insulin resistance is associated with cognitive function only among APOE4 negative participants, a relationship not observed in APOE4 carriers. There was no effect of central obesity on cognitive function. Longitudinal follow-up is expected to inform how metabolic risk factors may contribute to cognitive decline as the cohort ages.

INTRODUCTION

Over half of US adults > 64 years of age have "pre-diabetes" (American Diabetes Association, 2011). Both type 2 diabetes and pre-diabetes are characterized by insulin resistance (IR). Peripheral IR causes dysregulation of glucose, in turn causing damage to muscles, pancreas, kidney, heart, liver, and the nervous system. Risk of AD is also elevated in the presence of diabetes, in particular type 2 diabetes and hyperinsulinemia [236, 237]. The onset of type 2 diabetes is most common in middle age and risk for AD is elevated when diabetes occurs in middle age compared to old age; diabetes before the age of 65 corresponds to a 125 percent increased risk for AD [238].

According to the National Health and Nutrition Examination Survey (2009–2010), approximately 69% percent of Americans are overweight or obese (National Heart, Lung, and Blood Institute) Central obesity, also called visceral obesity, is associated with IR and increased risk for health conditions and cognitive dysfunction [239]. These two conditions may occur alone, but most often together. In addition to increasing risk for diabetes, heart disease and certain cancers, data now overwhelmingly point toward a role for IR and central obesity in cognitive decline and Alzheimer's disease (AD).[240-245]

Seminal epidemiological research indicates that vascular risk factors assessed at midlife (in participants ranging in age from 39-64 years; mean age = 50) predicts dementia 20 years later.[246] Obesity at midlife is associated with over a three fold increased risk for AD, while being overweight is associated with a two-fold increased risk.[247] The implication is that neural changes occurring in midlife position individuals on an aging trajectory which determines later cognitive outcomes.

Given that midlife may be a particularly vulnerable life-stage where cardiovascular risk factors are predictive of later cognitive decline, the current study assessed the relationship between insulin resistance, obesity, and cognitive function in a middle-aged cohort, the Wisconsin Registry for Alzheimer's Prevention. Primary analyses centered around four questions: 1) What is the relationship between insulin resistance and cognition? 2) How does adding obesity to the model affect that relationship? 3) Does gender interact with either of these metabolic predictors in affecting cognition? And 4) Do risk factors for Alzheimer's (family history or APOE4 status) interact with either metabolic predictor in influencing cognition?

Primary analyses centered around four questions: 1) What is the relationship between insulin resistance and cognition? 2) How does adding obesity to the model affect that relationship? 3) Does gender interact with either of these metabolic predictors in affecting cognition? 4) Does FH or APOE-e4 status interact with either metabolic predictor in influencing cognition? We hypothesized that higher insulin resistance and central obesity would be associated with lower cognitive function.

METHODS

Participants. The Wisconsin Registry for Alzheimer's Prevention (WRAP) is an ongoing longitudinal study of a sample of middle-aged adults enriched for a family history of Alzheimer's disease (Sager et al., 2005). Recruitment began in 2001 and is ongoing. WRAP participants were generally between the ages of 40 and 65 years at baseline, English speaking, and had a parent with either autopsy-confirmed or probable AD (FH+) as defined by NINCDS-ADRDA research criteria (McKhann et al., 1984) or no parental history of AD or other dementia (FH-) [182][13] FH+ subjects were volunteers whose parent(s) had been evaluated in a memory assessment clinic at the University of Wisconsin-Madison or at affiliated satellite memory assessment clinics, and others who learned about

the study from educational presentations or word of mouth. To verify the diagnosis of AD in parents not directly assessed, autopsy reports or parental medical records were reviewed. Most FH- participants had mothers who survived to at least age 75, and fathers to at least age 70, without Alzheimer's disease, other dementia, or significant memory deficits. These participants were recruited through community presentations and word of mouth.

The WRAP sample currently includes 1536 middle-aged adults recruited predominantly in the upper Midwest and tested at sites in Madison, LaCrosse, and Milwaukee, Wisconsin. Data for the present analyses were taken from Wave 2 of the study, the first wave for which fasting blood glucose and insulin data were available (N=1120). Our analyses excluded subjects who presented with AD (N=1) or other neurological conditions that can impair cognition, including stroke, epilepsy, meningitis, Parkinson's disease, and multiple sclerosis (N=83), as well as subjects with incomplete data on any outcomes or predictors of interest (N=61), leaving N= 975 subjects in the final sample.

General study procedures. Baseline assessment included a battery of commonly used clinical neuropsychological tests (see Sager, Hermann, & La Rue, 2005, for a description of the cognitive battery), completion of questionnaires about health history and lifestyle, laboratory tests, and APOE genotyping. All study procedures have been approved by the Health Sciences IRB of the University of Wisconsin-Madison.

Cognitive outcomes. Factor analysis using promax rotation and maximum likelihood estimation (Grice, 2001) were used to reduce the set of cognitive measures to a smaller number of factors and obtain weights used to combine the measures within each factor. The resulting weighted factor scores were then standardized (~N (0, 1)) into z-scores, using means and standard deviations obtained from the whole baseline sample (additional details on the factor analysis methods used for the WRAP sample can be found in Dowling et al., 2010). Among tests begun at Wave 1, there are two factors representing new

verbal learning and recall (Immediate Memory; Verbal Learning and Memory), both derived from the *Rey Auditory Verbal Learning Test* (Lezak, Howieson, & Loring, 2004, and two factors reflecting components of executive function (Working Memory; Speed and Flexibility). A similar factor analysis was performed on Wave 2 data, suggesting that the newly-added Brief Visuospatial Memory Test required a separate factor. Table 1 shows the five factors and the cognitive tests that loaded on each factor.

Insulin resistance. To assess insulin resistance, venous blood was collected in the morning after a 12h fast. Blood samples were collected in Cell-free plasma/serum was aliquoted into 1.5mL micro centrifuge tubes and frozen at -80 degrees Celsius. Plasma and serum samples were analyzed at the University of Wisconsin Hospital and Clinics Hospital Laboratory (Madison, WI). To assess fasting glucose, plasma was assayed using hexokinase glucose method (Siemens Dimension Vista). To assess fasting insulin, serum was assayed using chemiluminescent immunoassay on an ADVIA Centaur XP Immunoassay System (Siemens Corporation, Washington DC, USA). Insulin resistance was calculated from fasting serum insulin and fasting plasma glucose using the homeostatic model assessment of insulin resistance (HOMA-IR) method (Matthews et al., 1985) calculated as HOMA-IR = Insulin (mg/dL) x Glucose (uIU/mL) / 405.HOMA-IR was log-transformed and standardized to approximately N (0,1) for ease of interpreting model coefficients.

Obesity. Central obesity was defined based on waist measurements. We used separate thresholds for men and women, as described in The National Cholesterol Education Program Adult Treatment Panel III[248]Men were less likely to be categorized as obese than were women (Men: 113 (38%) obese; Women: 323 (48%) obese). In models, obesity was represented as a categorical variable (1=obese).

Diabetes. Diabetes was diagnosed in participants (N= 52) who had a fasting blood glucose measurement greater than 125 (N=20), reported being on one of several antidiabetic medications (N=16), or both (N=16) .

Statistical analysis. Analyses were conducted using SAS (version 9.3). Outcomes were approximately normal (|skewness| & |kurtosis| < 1).

We used linear mixed models to examine relationships between cognition, metabolic predictors, sex, and familial/genetic risk. A random intercept was included to account for sibling clusters. Major predictors of interest were HOMA-IR and obesity. Also considered were interaction effects between those two predictors and between each of those predictors and sex, FH, and APOE-e4 status. Age and education level (highest degree attained) were included as covariates. In modeling each outcome, interaction effects with p>.10[2] were rejected and the model was refit iteratively until a final model was obtained for that cognitive outcome. Collinearity diagnostics were examined and models were rerun omitting correlated predictors if tolerances fell below 0.2 (CITE Cohen Cohen West & Aiken). To reduce model overreliance on outliers, significant effects of interest were further explored in secondary analyses using Winsorized predictors (CITE) and influence diagnostics. Finally, models were rerun excluding diabetics to determine the contribution of frank metabolic disease to any observed effects. Because our design was unbalanced, we used the Kenward-Roger approximation to calculate denominator degrees of freedom for all tests (Schaalje, McBride, & Fellingham, 2002). For significance testing, we set α=.05.

Results

Our final sample included data from 975 participants. Demographic and other characteristics of our sample can be found in Table 2. Participants included in the analyses were similar to excluded participants in terms of all predictors and

[2] While a p-value between .05 and .10 is not statistically significant, we chose to keep interaction terms in this range in the model to ensure that our interpretation of related main effects was conservative.

outcomes of interest[3], with the exception that excluded participants performed marginally more poorly on the Visual Learning factor ($t(1017) = -1.87$, $p = .062$).

Aim 1: Insulin resistance

In the first stage of model building, we examined the effect of HOMA-IR and its interaction with age on cognitive outcomes. We found a significant negative effect of HOMA-IR on Speed & Flexibility ($\beta = -0.065$, $F(1,964) = 5.09$, $p = .024$). All other main effects of HOMA-IR were non significant ($p > .05$), as were all interactions of HOMA-IR with age ($p > .10$). Examination of model tolerances suggested no multicollinearity (all tolerances > 0.6). Secondary analyses of Speed & Flexibility using Winsorized HOMA-IR and excluding influential outliers produced substantially similar results. However, when diabetics were excluded, no effects of HOMA-IR were significant ($p > .05$). Figure 1 illustrates the bivariate relationships between HOMA-IR and Speed & Flexibility both including (dashed) and excluding (solid) diabetic participants.

Aim 2: Insulin resistance and obesity

In the second stage of model-building, we added terms representing the main effect of obesity and its interaction with HOMA-IR. All interaction terms were non significant ($p > .10$). Furthermore, in no model was there a significant main effect of obesity on cognitive performance ($p > .10$). When controlling for obesity, the main effect of HOMA-IR on Speed & Flexibility was similar in magnitude, though now only marginally significant ($\beta = -0.064$, $F(1,962) = 3.62$, $p = .057$). Examination of model tolerances suggested no multicollinearity (all tolerances > 0.6). Secondary analyses of Speed & Flexibility using Winsorized HOMA-IR and excluding influential outliers produced similar, marginally-significant results. Again, when diabetics were removed, no effects of interest were significant.

[3] Because metabolic variables began to be collected at Wave 2, excluded participants were disproportionately likely to be from our most recent, most ethnically diverse site, and therefore disproportionately likely to be nonwhite.

Aim 3: Interactions with sex

In the final stage, interaction terms were added to each model to explore whether sex affects the predictive relationships between HOMA-IR, obesity, and our five cognitive outcomes. In all models, these interaction terms were nonsignificant ($p > .10$).

Aim 3: Interactions with familial risk

In the final stage, interaction terms were added to each model to explore whether FH and APOE affect the predictive relationships between HOMA-IR, obesity, and our five cognitive outcomes. For speed & flexibility, both interactions with obesity were nonsignificant. However, we observed a significant APOE by HOMA-IR interaction ($F(1,944) = 7.00$, $p = .0083$) and a marginal FH by HOMA-IR interaction ($F(1,955) = 2.92$, $p = .088$). To explore simple main effects, separate mixed models were run for ε4- and ε4+ groups, and these suggested that the effect of HOMA-IR was significant for ε4- participants only (ε4-: $\beta = -0.11$, $F(1,586) = 6.55$, $p = .011$; ε4+: $\beta = .0074$, $F(1,361) = 0.02$, $p = .89$). In both submodels, the FH by HOMA-IR interaction was nonsignificant and was removed. Collinearity values were just above the common threshold of 0.2. When the marginal FH by HOMA-IR interaction was removed, tolerances improved considerably, and parameter estimates of interest were unchanged. Similarly, neither approach to influential datapoints (removing or Winsorizing) changed results substantially. As in earlier analyses, in models of the remaining four outcomes, none of the interaction effects of interest were significant ($p > .10$).

DISCUSSION

Insulin resistance, diabetes and obesity have been linked to cognitive decline and greater risk for developing dementia and the risk for dementia appears to be increased when symptoms are present at midlife. The current study found that insulin resistance only had an effect upon cognition when

participants with the greatest disease severity (i.e. diagnosed diabetes) and APOE4 negative individuals were considered. There were no significant effects of elevated central obesity on cognitive function.

Interestingly, cognitive effects were found in speed and flexibility, rather than memory function. Accumulating evidence demonstrates the importance of insulin in memory formation, including a small number of studies that show insulin mediated glucose uptake in the medial temporal lobe and hippocampus and improved memory function following acute rises in insulin(REF). Studies demonstrating improvements in memory function after acute insulin compared AD, MCI and controls showing the greatest improvements in the patient population. Our population has not developed severe memory deficits as those with Dementia due to Alzheimer's, which may account for lack of finding regarding fasting insulin levels. The test components of Speed and flexibility score include trails making A&B and the stroop-interferance trial, which can infer speed and flexibility as well as executive function. Executive function can be described as cognitive abilities responsible for the top-down control of decision-making, planning, self-monitoring, behavior initiation, organization and inhibition[249]. Decline in executive function in AD is becoming more and more apparent that AD patients are impaired on a variety of executive function tasks[249, 250]. Abbtecola, Paolisso et al examined insulin resistance older persons and found IR correlated with measures of executive function even after adjusting for age, sex, years of education and a panel of cardiovascular risk factors including but not limited to body mass index, triglycerides, and hypertension[251]. In addition. The third component of the speed and flexibility test include stroop interference task, which is widely known to assess working memory. Working memory is the cognitive function responsible for keeping information afoot, manipulating it, and using it in current thinking or a means to delegate tasks or information encountered to parts of your brain that can take

action[252]. Our study provides information about cognitive domains affected by insulin resistance in a cohort of cognitively healthy individuals enriched for AD.

Insulin has more than simply a role in the glucoregulatory control in the brain. Insulin receptors or distributed through the brain and highest in the hippocampus, amygdala, and parahippocampal gyrus; intermediate in cerebellum, cerebral cortex, and caudate nucleus[253] We can only speculate about the underlying biological mechanisms preceding the midlife cognitive changes presented in this study. An insulin resistant state has been associated with white matter lesions and such lesions have been associated with lower cognitive functions[254]. In human studies, differences in cerebrospinal fluid levels of tau suggest a role for tau dysregulation in the decline of cognitive function specifically processing speed and flexibility. Greater levels of IR are associated with alteration in white matter microstructural integrity in cognitively healthy middle aged and older adults[195]. White matter health is important for salutatory conduction of the neuronal signals across the brain and is generally composed of myelin-coated axons[255]. Disruption of white matter can affect the transmission of neuronal signals from one cell to another manifesting as decreased speed of processing and transmission across brain networks[256]. Further IR has been implicated in tau hyperphosphorylation, leading to dysregulated dissociation of tau from microtubules and potential axonal dysfunction[257, 258]. Significantly, animal studies of diet induced insulin resistance have found application of Pioglitazone, a diabetes drug that makes cell more sensitive to insulin, leads to reduce tau phosphorylation in APOE ε3 trangenic mice but not in the APOEε4 transgenic animals. The results imply once again a more significant effect of IR in APOEε4 negative animals[259]. Alteration in white matter and axonal health may converge to decrease processing speed in middle-age.

Of note, significant findings went away when those with Diabetes were excluded from the sample. Chronic elevations in peripheral insulin such as those which might be found in Diabetes Mellitus II (DMII), can have detrimental effects, including adverse effects on brain synaptic plasticity. DMII Studies assessing DMII and cognition report significant changes in processing speed and executive function. The pathophysiology of DMII is far more reaching than simply endocrine dysregulation of insulin and includes microvascular dysfunction, abnormal glucoregulatory control, amyloid deposition, dyslipediemia, inflammation, and changes in blood-brain barrier permeability[260] . Even though IR did not by itself show a significant association with cognitive function it is important to note that there is a trend. Also, including those with diabetes may be a more representative sample of the general population.

Findings were significant in participants without APOE4. Suggests effect may be independent of other AD-linked mechanisms, or that APOE4 influences cognitive function, resulting in "ceiling" effect were effect of IR couldn't be seen. Only a handful of studies have assessed the relationship between IR and cognition at middle age and even less explored the effects of APOE4 and family history status. However, information regarding IR effects on cognition as modulated by APOE4 has been explored in therapeutic studies comparing AD patients with age-matched controls. In vivo evidence of differences in insulin-mediated energy metabolism between epsilon4 and non-epsilon4 AD, and suggest that defective insulin action may be of particular pathophysiologic significance for patients without an epsilon-4 allele[261]. Further, Insulin administration facilitated recall on two measures of verbal memory in memory-impaired epsilon4- adults, with performance generally peaking at 20 IU. In contrast, memory-impaired epsilon4+ subjects demonstrated a relative decline in verbal memory. Insulin also differentially modulated plasma amyloid-beta for memory-impaired subjects and normal controls, effects that again differed by

APOE genotype. These findings suggest that groups with different genetic risks for AD may show differential dose-response curves following intranasal insulin administration[219]. In summary, IR may be preferentially detrimental in the development of cognitive dysfunction in APOE4- subject. Although we did not see memory deficits with peripheral IR our results support the growing evidence of an increased role of IR in APOE4- individuals compared to APOE+ in the development of cognitive dysfunction.

Somewhat surprising was the lack of effect of central obesity on cognitive function. Indeed, even when the highest quartile of obese participants was examined, we found no significant effects. One possibility is that only after prolonged central obesity that effects on cognition become manifest. Another possibility is that factors such as inflammation or triglyceride levels need to be taken into account, as some individuals who are overweight, may not exhibit the full spectrum of metabolic dysfunction found in other patients[262].

Conclusion

Taken together, the results suggest that insulin resistance is associated with cognitive function only when participants with clinically diagnosed glucoregulatory dysfunction are included in the sample. Furthermore, the results indicate that insulin resistance is associated with cognitive function only among APOE4 negative participants, a relationship not observed in APOE4 carriers. There was no effect of central obesity on cognitive function. Longitudinal follow-up is expected to inform how metabolic risk factors may contribute to cognitive decline as the cohort ages.

Table 1.

Factor structure of five cognitive domains identified in the WRAP battery.

Factor name	Cognitive test
Immediate Memory	Rey Auditory Verbal Learning Test – Trials 1 & 2[a]
Verbal Learning & Memory	Rey Auditory Verbal Learning Test – Trials 3 through 5 & delayed recall
Working Memory	Digit Span Forward, Digit Span Backward, and Letter-number Sequencing (Wechsler Adult Intelligence Scale-III)[b]
Speed & Flexibility	Stroop Color-Word Test – interference trial[c]
	Trail-Making Test – Parts A & B[d]
Visual Learning & Memory	Brief Visuospatial Memory Test

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[a]Lezak, Howieson, & Loring, 1984

[b]Wechsler, 1999

[c] Trenerry, Crosson, DeBoe, & Leber, 1989

[d]Reitan & Wolfson, 1993

Table 2.

Characteristics of the WRAP sample (N=975). At right, correlations between variables of interest and HOMA-IR. Unless indicated, Spearman correlations are reported.

	Sample characteristics	$r_{HOMA-IR}$
Age, mean (SD)	57.7 (6.6)	.06†
Site, N (%)		
Madison	756 (77)	
La Crosse	202 (21)	-.050
Milwaukee	17 (2)	.06
Sex, number female (%)	676 (69)	-.17
Family history, number FH+	717 (69)	.037
APOE, number ε4+ (%)	378 (39)	.030
Race, number white (%)	957 (98)	-.040
Education, number with BA (%)	604 (62)	-.08
Full-scale IQ, mean (SD, range)	113 (9)	-.0029†
HOMA-IR, mean (SD)	2.6 (3.7)	
BMI, mean (SD)	28.8 (6.3)	.37†
Waist, number obese (%)	436 (45)	.48

† Pearson correlation.

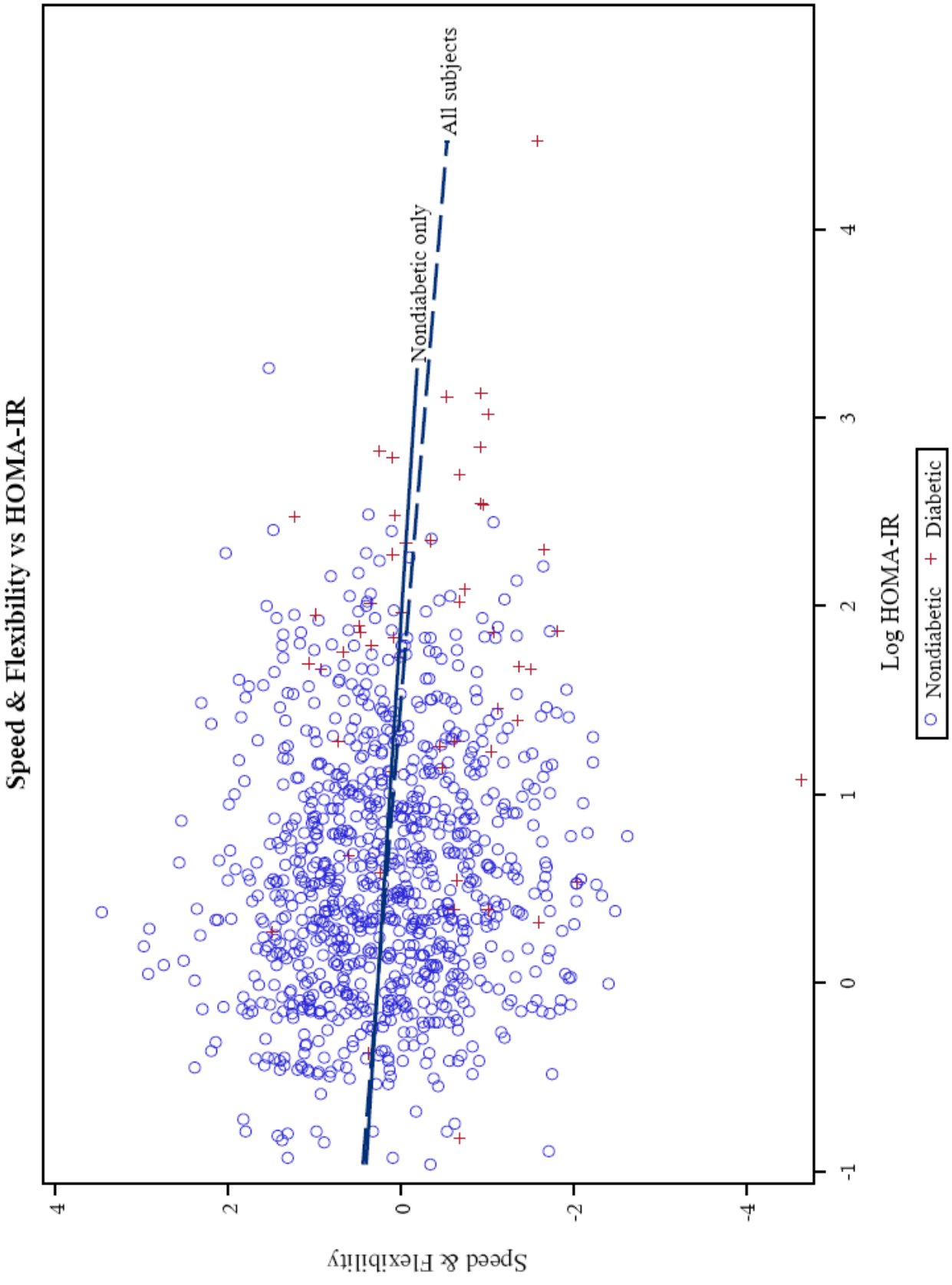

Figure 1. Bivariate relationship between HOMA-IR and Speed & Flexibility.

Probably only want to note those that participants were actually taking.

List of antidiabetic medications qualifying participants for a diabetes diagnosis.

Metformin

Glimepiride

Glyburide

Glipizide

Micronized glyburide

Repaglinide

Nateglinide

Pioglitazone

Sitagliptin

Saxagliptin

Linagliptin

Acarbose

Miglitol or meglitol

Colesevelam

chlorpropamide

Pramlintide

Rosiglitazone

Tolbutamide

Tolazamide

Acetohexamide

Exenatide

Alogliptin

Saxagliptin

Alogliptin + metformin

Alogliptin + pioglitazone

Pioglitazone +
metformin
Glyburide + metformin
Glipizide + metformin
Sitagliptin + metformin
Saxagliptin + metformin
Repaglinide + metformin
Pioglitazone +
glimepiride
rosiglitazone +
metformin
linagliptin + metformin
sitagliptin + simvastatin
Insulin
Aspart

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Appendix B

Central Obesity correlates with decrease in Cerebral Blood Flow as measured by Arterial Spin Labeling

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Abstract

Background- Obesity at midlife increases the risk of developing dementia later in life. Further, obesity is strongly correlated with cardiovascular risk factors, cognitive decline, and dementia. The mechanism behind which obesity affects cognitive decline is still unknown. Surprisingly there are limited studies evaluating the effects of central obesity on cerebral blood flow. In our study, we examined the relationship between obesity and cerebral blood flow (CBF) in cognitively healthy late middle-aged adults enriched for Alzheimer's disease risk factors.

Methods and Results- Late middle-aged adults from the Wisconsin Registry for Alzheimer's Prevention (*N*=124, mean age=60.7) underwent a fasting blood draw, arterial spin labeling perfusion MRI, and neuropsychological testing. Obesity was defined as a male's waist being greater than 102cm and a woman's being greater than 88cm.

Univariate analyses were conducted on (#) Regions of interest controlling for age, sex, and hypertension to examine if obesity was associated with decreased CBF in areas significant to Alzheimer's Disease.

Results

We found significant effects of central obesity where there was decreased CBF in the temporal, parietal, and precuneus areas. (Need to add each area and the p values)

Conclusions- In our sample of 124 late middle-aged adults we find decreased CBF to brain regions known to be affected by AD, in obese compared to non-obese participants. Our findings suggest a significant effect of obesity on the vascular health of non-demented adults enriched with risk factors for Alzheimer's disease.

Introduction

It is estimated that 78.6 million U.S. adults are obese and $147 billion U.S. dollars were spent in 2008 on obesity medical costs [305]. Obesity is playing a central role in today's healthcare industry, and American lives are at risk. Central Obesity (CO) is associated with many metabolic risk factors such as hypertension, high triglycerides, low HDL cholesterol, inflammation, and insulin resistance; it is also associated with cognitive decline and Alzheimer's Disease (AD)[306-309]. CO is a more statistically accurate measure compared to body mass index (BMI) or other obesity measures when predicting cardiovascular risk factors and metabolic risk factors[310-312]. Moreover, a large portion of the United States' population is aging, increasing the incidence of age related health concerns such as dementia and (AD) [305]. The underlying cause of sporadic AD is still to be discovered. Obesity at midlife increases the risk of developing Alzheimer's dementia later in life. It is unclear of the association between midlife metabolic disturbances and AD. This study aims to shed light on the role of obesity in the increased risk for AD. Several studies suggest that midlife may be a critical period when cardiovascular risk factors influence cognitive aging

trajectories[48, 306, 313, 314]. Midlife is a critical period as interventions could take place at this time period to decrease the risk of declining into Alzheimer's dementia.

Many mechanisms have been proposed linking cardiovascular risk factors and obesity to cerebral function, however little is known about obesity alone, its direct effects on the brain, and the midlife changes that occur. Some proposed mechanisms include polymorphism of cholesterol derived APOE gene, impaired insulin-signaling, hyperhomocysteinemia, inflammatory cytokines, and decreased neurogenesis (REF). Risk factors of obesity such as abnormal triglycerides, HDL, and blood pressure have been linked to decrease in cerebral blood flow[315-318]. Furthermore, recent studies have conveyed decreased global and regional cerebral blood flow (CBF), in those with mild cognitive impairment and Alzheimer's Disease, suggesting that hypoperfusion may be a possible mechanism for neural damage and cognitive decline [306, 318].

Therefore, the objective of the current study was to assess if obesity alone is associated with CBF differences in midlife and determine if cerebral perfusion defines part of the mechanism for cognitive decline. We were interested in the regions associated with AD, specifically areas of the hippocampus, temporal and parietal regions, the cingulum, and basal forebrain. We hypothesized that

centrally obese individuals would have lower CBF in regions significant to AD than those who were not classified as obese.

Methods

Subjects:

One hundred twenty-four participants were recruited from the Wisconsin Registry for Alzheimer's Prevention, WRAP, a registry of cognitively normal adults enriched for family history of late onset AD (21). Members with family history are defined by one or both parents with autopsy-confirmed or probably AD defined by NINCDSADRDA criteria (22). The selected subjects consisted of: normal cognitive function determined by neuropsychological evaluation (mmse), no contraindications for MRI and a subsequent normal MRI scan, no current diagnosis of major psychiatric disease or other medical conditions (e.g., myocardia infarction, or recent history of cancer), and no history of head trauma. All participants underwent a fasting blood draw, MRI, and neuropsychological testing. Subjects were selected based on those who had a 20-25sec delay scan time of Arterial Spin Labeling image and passed a quality assurance evaluation by a research assistant and a research specialist. If there was more than one MRI visit, we selected the scan that was closest to the medical exam date. One subject was excluded due to a waist measurement that fell outside two standard deviations of the cohort. Subject demographics can be found in Table 1. The

University of Wisconsin Institutional Review Board approved all study procedures and each participant provided signed informed consent before participation.

Central Obesity

Waist Circumference was measured at the smallest circumference of the waist. Central Obesity trhesholds were determined and binarized by males having a waist >102 cm and females having a waist > 88cm (1).

Brain Imaging Acquisition

MR scanning was performed on a General Electric 3.0 Tesla Discovery MR750 (Waukesha, WI) MRI system with an 8-channel head coil and parallel imaging (ASSET).

Resting CBF assessments were made using background-suppressed pseudo-continuous ASL (pcASL)(23, 24), featuring a 3D fast spin echo spiral sequence that utilizes a stack of variabledensity spiral 4ms-readout and 8 interleaves. Scan parameters were TR = 6000 ms; TE = 21 ms; FOV = 240 x 240 x 160 mm; slice thickness = 4 mm no gap; matrix size=128 x 128; NEX=3; and labeling RF amplitude=0.24 mG. Multi-slice spin labeling was implemented using a single coil that eliminates off-resonance errors (25). The pcASL scan included 3 averaged acquisitions, each consisting of a control image subtracted from a labeled image. The sequence also included a fluid-suppressed proton

density (PD) acquisition, with the same imaging sequence/image slab location as the pcASL but without the RF labeling preparation, for CBF flow quantitation and image registration. In order to reduce variability in the CBF assessment, participants fasted for a minimum 4-hours prior to scan, abstaining from food, tobacco, caffeine, and medications with vasomodulatory properties. We have previously reported excellent test-retest reliability ($r > 0.95$) of this pcASL procedure (26).

ASL Processing

The T1-weighted volume was segmented into tissue classes using the updated segmentation feature in SPM12 (www.fil.ion.ucl.ac. uk/spm). The segmentation procedure produced a deformation field mapping the T1 image to Montreal Neurological Institute (MNI) standard space.

The CBF images were processed using SPM12. Each participant's co-localized PD image to their T1-weighted image and the derived transformation matrix was applied to the CBF map.

Regions of interest (ROIs) were selected from the Automated Anatomical Labeling (AAL) atlas (28). These ROIs included the supramarginal gyri, middle and superior temporal gyri, hippocampus, precuneus, and posterior cingulate. The inverse deformation field created during the tissue segmentation was applied to each of the selected AAL ROIs to produce ROI masks in native space. To further constrain ROI analyses to gray matter (GM), the ROI masks

were then multiplied by the binarized GM probability map thresholded at 0.3.

Statistical Analyses

Analyses were conduced with IMB SPSS version 22.0 (Chicago, IL). Differences in demographics, clinical measures, and covariates were analyzed for those who were obese and those who were not obese using ANOVA t tests and X^2 tests as appropriate. Results are outlined in Table 1 and examined at $p < 0.05$.

To account for inter-individual differences in ASL scan signal, the raw ROI value was scaled by dividing by a neutral region. We define the neutral region as a region that is not typically associated with AD. Further, we aimed to define a region without significant effects of central obesity. Thus, an average of the lingual gyri was used as our neutral region.

Univariate analyses were carried out to test if central obesity was associated with decreased perfusion in scaled regions of interest outlined in Figure 1 controlling for age, gender, and hypertension.

Results

There were no significant differences between the obese and control group on gender, age, hypertension, baseline cognitive score (mmse), or years of education. Participant demographics between obese and controls are given in Table 1.

Table 1. Participant demographics

Demographic		All N=124	Not Obese=71	Obese=53	Statistic	p
Women (%)		87(70.2)	45(63.3)	42(79.2)	X^2=3.649	0.074*
Age, M (SD)		60.7(6.1)	60.9(5.8)	60.5(6.43)	F=.158	0.692
Hypertension (%)	60(48.4)	29(40.8)	31(58.5)	X^2=3.78	0.069*	
mmse M(SD)	29.4(.96)	29.5(.95)	29.4(.965)	F=.441	0.508	
Education, M (SD)	16.5(2.2)	16.62(2.1)	16.23(2.4)	F=.942	0.334	

Statistical analysis yielded significant findings of decreased CBF in temporal, parietal, and precuneus areas in the obese group outlined in Table 2 and illustrated in Figure 2, specifically, the right supramarginal gyrus (F=7.42, p=0.007), right middle temporal gyrus (F=5.71, p=0.018), the left precuneus (F=10.13, p=0.002), the right precuneus (F=10.28, p=0.002), the right angular gyrus (F=5.85, p=0.017), and the left angular gyrus (F=16.00, p=0.000).

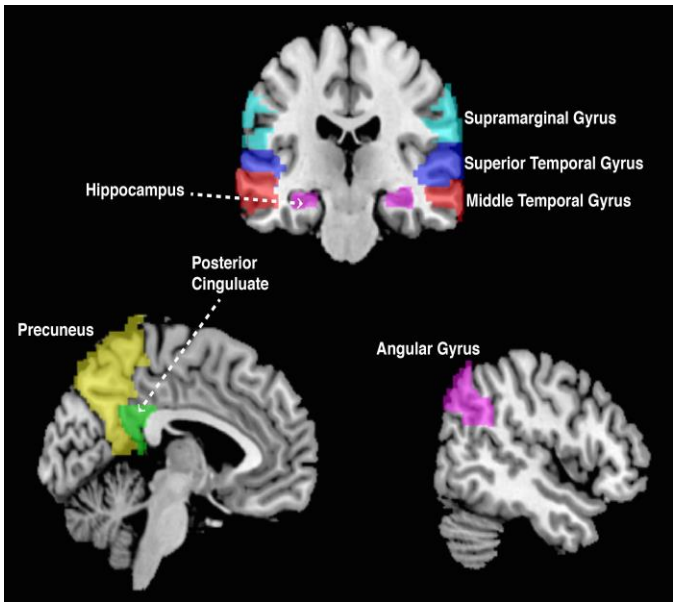

Figure 1. Regions of interest included in the regression model

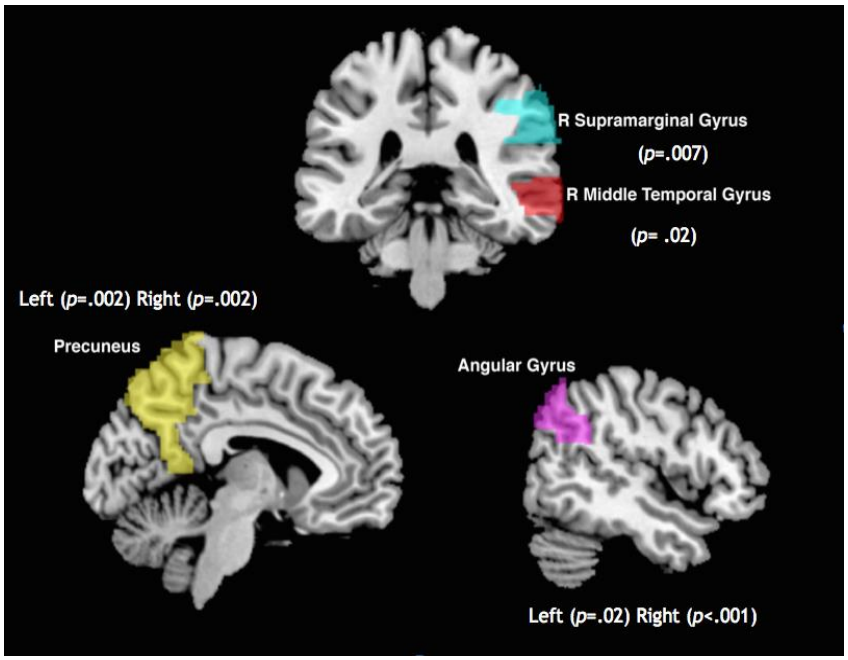

Figure 2. Regions of interest with a significant association with central obesity

Table 2. Statistical univariate tests controlling for age, gender, and hypertension temporal, parietal, and precuneus

Results	F	p
Supramarginal L	1.34	0.249
Supramarginal R	7.42	**0.007
Middle Temporal L	1.12	0.292
Middle Temporal R	5.71	*0.018
Superior Temporal L	0.007	0.932
Superior Temporal R	2.45	0.121
Hippocampus L	0.053	0.819
Hippocampus R	0.984	0.323
Precuneus L	10.13	**0.002
Precuneus R	10.28	**0.002
Posterior Cingulum L	1.97	0.163
Posterior Cingulum R	1.20	0.275
Angular L	5.85	*0.017
Angular R	16.00	**0.000

Discussion

Central obesity contributes to many health risks linked to vasculature health [315-318]. In addition, obesity at midlife increases the relative risk of developing AD later in life. Biological mechanisms to explain this increased risk have not been fully explored. Studies have reported altered CBF in those with mild cognitive impairment and AD [306].In our study we found decreased cerebral blood flow in a cohort of middle aged cognitively healthy individuals who are collectively enriched for AD risk factors. Univariate analyses revealed obese individuals who had decreased CBF while controlling for age, sex, and hypertension. Significant areas of decreased CBF included the precuneus, angular gyri, right supramarginal gyrus, and right middle temporal gyrus.

These highlighted areas are key in AD behavioral manifestation. The precuneus is involved in self-centered mental imagery and visual episodic memory retrieval [319]. Moreover, the angular gyri are vital in language, memory retrieval and attention [320, 321]. When these areas are stricken by pathology, normal cognition and memory function are at risk. The supramarginal gyri, also a player in memory, presents symptoms of AD when compromised [322]. Lastly, the function of the right middle temporal gyrus is largely unknown, however studies suggest atrophy predicts a decline to AD [323].

Obesity is a major cardiovascular risk factor[324]. Many mechanisms have been proposed for linking cardiovascular risk factors and obesity to cerebral function, however effects aside from vasculature may have an impact with only

some of them being identified. Obesity and cardiovascular risk factors influence cerebral function through decreased cerebral utilization of glucose, hypercholesterolemia, amyloid and plaque burden, insulin signaling, vitamin D, and hypertension[325-329].

We chose to investigate the effects of obesity while controlling for hypertension. Since we are particularly interested in cerebral blood flow, we chose to control for hypertension, as this is a main determinant of blood flow. Occlusion and atherosclerosis in the carotid system and the vertebral-basilar system could also affect blood flow, however we are particularly interested in the flow in the cranial cavity [330]. Thus, controlling for hypertension in this study allows opportunity to consider other mediating mechanisms in the brain relating to decreased CBF. We can conclude that obesity has effects on blood flow at midlife in areas affected by AD.

The regions affected are anatomically most distal to their main artery of supply. The precuneus is most distal to the anterior cerebral artery; the angular gyri, right supramarginal, and middle temporal gyri are more distal to the middle cerebral artery[331]. Cells may be at a disadvantage when acquiring nutrients based on their anatomical location in reference to their main cerebral arteries. Evidence to support the vascular changes comes from Selim et al who found BMI was negatively associated with decreased flow velocity, and positively associated with cerebrovascular resistance in the middle cerebral artery of obese participants. These finding were independent of diagnosis of

diabetes mellitus, hypertension or stroke[332]. Further, obesity is associated with microvascular impairment, which may explain the decreases in blood flow predominately at the ends of major arteries as opposed to areas that are more proximal to the larger vessels. In addition, obesity is also associated with abnormalities in microvascular patterns, reduced small vessel density, inflammation and impaired endothelial function and vascular reactivity [333, 334] in peripheral and possibly even in central vascular beds.

Obesity at midlife increases the risk of developing AD later in life, and molecular mechanisms at midlife at are currently under investigation. Molecular frontrunners include the association of obesity with insulin resistance, hyperlipidemia, endocrine abnormalities, and overall poor cardiovascular health. Abdominal obesity has been shown to have visceral effects on the abdominal organs causing irritation and release of hormones associated with the gastrointestinal organs. These hormones can cause changes in metabolism specifically related to adipose tissue. In insulin resistance, the signals normally meant to allow for storage of plasma blood, are not recognized due to persitenant hyperinsulinemia leading to a down regulation of the insulin receptor. These signals include activation of the Pi3 kinase and downstream to that the anabolic lipid enzymes.

Obesity is linked to decreased brain volumes. Meaning there is just less tubing for the blood to flow through, further studies on brain volumes at middle age will need to be done. Moreover, possible increased intracranial pressure due

to the enclosed nature of the skull, neuroprotective hormone abnormalities, and CO related inflammation might be depriving these areas of blood supply, decreasing the delivery of nutrients necessary for cell function[335-337]. Conversely, obesity induced neuronal atrophy, calls for less cell nutrients, resulting in decreased local blood flow [338, 339].

A few limitations should be mentioned. This study recruited participants from the Wisconsin Registry for Alzheimer's Prevention and may limit the generalization of the results as these participants are enriched for a family history of AD. Moreover, this study is cross-sectional and doesn't hold as much power as if a longitudinal study was conducted for this analysis. Sample size is also a limitation. Lastly, the pulse delivered in the MRI may be affected with the neck size of the participant, altering the signal produced after the delay period. Future studies should take into account the neck circumference when participants receive their scan.

This was the first study examining effects of central obesity in a large cohort of cognitively healthy individuals at increased risk for AD. Therefore, this study proposes that obesity predicts decreased cerebral blood flow especially at the ends of the arterial supply, which is important for cognitive function and health.

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