FOOD FOR THOUGHT: EXPLORING MULTIGENERATIONAL EFFECTS OF A COMPREHENSIVE MEDITERRANEAN DIET ON COGNITION AND BIOMARKERS OF ALZHEIMER'S DISEASE

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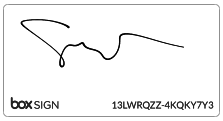

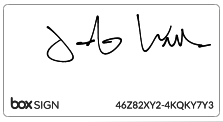

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CHAPTER 1: GENERAL INTRODUCTION

1. Introduction to Alzheimer's Disease

Approximately 10.7% of Americans over the age of 65 have been diagnosed with Alzheimer's Disease (AD), the most prevalent form of dementia ("2023 Alzheimer's disease facts and figures," 2023). Although death rates for several diseases, like heart disease, have decreased in the U.S. over the last twenty years, AD death rates have rapidly increased by about 145% since the year 2000 ("2021 Alzheimer's disease facts and figures," 2021). AD rates are not only increasing in the U.S., but all over the world. Historically, researchers have reported that AD prevalence is typically lower in countries boarding the Mediterranean Sea, like Greece. For example, prior findings from the Hellenic Longitudinal Investigation of Aging and Diet (HELIAD) study in Greece revealed that 5% of Greek adults over the age of 65 had dementia, and AD accounted for 73.5% of total dementia cases in this population (Kosmidis et al., 2018). However, recent updates from this longitudinal study have revealed that AD prevalence has increased in Greece over the last few years, likely due to modern lifestyle changes, such as novel dietary patterns (Vlachos et al., 2021). For example, Western foods have become more accessible in Mediterranean regions, and thus, less people are adhering to the healthy, traditional Mediterranean diet. Sadly, this dramatic worldwide increase in AD rates is not expected to slow down in the near future. For example, about 6.7 million Americans over the age of 65 currently have AD, and this number is estimated to double in approximately forty years ("2023 Alzheimer's disease facts and figures," 2023).

The progression of this fatal disease is known as the Alzheimer's disease continuum, and it is divided into three stages: preclinical AD, mild cognitive impairment (MCI), and dementia ("2023 Alzheimer's disease facts and figures," 2023). Initially, AD patients have MCI, but, as the disease progresses, symptoms continually worsen, and patients begin to experience difficulties with everyday tasks. Altogether, AD patients suffer from memory loss, mental confusion, and

changes in behavior and personality ("2023 Alzheimer's disease facts and figures," 2023; Bekris et al., 2010). Finally, the severe stage of AD induces tremendous memory loss, along with both cognitive and physical disabilities. During this final stage, AD patients struggle with basic physiological functions, such as talking, eating, drinking, and walking. Additionally, at this final stage in the disease, the AD brain is immensely injured in several different regions that regulate crucial cognitive processes, movement, and essential reflexes, like swallowing ("2023 Alzheimer's disease facts and figures," 2023). As there is currently no cure for AD, the most severe stage ultimately results in patient death.

Not only does AD affect the individual patients, but also their caretakers, as they require constant care from healthcare workers and family members. The continual physical and psychological burden of being an AD patient caretaker is extremely stressful, such that 59% of caretakers have reported that they feel highly or very highly emotionally stressed ("2021 Alzheimer's disease facts and figures," 2021). Thus, it is imperative for researchers to further explore potential prevention strategies and more effective therapies to diminish AD prevalence, and the terrible suffering forced upon patients, caretakers, and their families.

1a. Hallmark Pathologies of AD

AD was first discovered in the early 1900's by Dr. Alois Alzheimer (Alzheimer et al., 1995). While conducting a postmortem autopsy, Dr. Alzheimer identified two, key hallmark pathologies of AD, including amyloid beta (Aβ) plaques and hyperphosphorylated neurofibrillary tau tangles (Alzheimer et al., 1995; Hippius & Neundörfer, 2003). These hallmark pathologies primarily develop in the cortex and hippocampus, two brain regions that are responsible for the neural mechanisms of learning and memory. Thus, these pathologies disrupt crucial learning and memory processes in the AD brain. Initially, Aβ plaques develop in the

cerebral cortex, and subsequently advance in the hippocampus (Alzheimer et al., 1995; Hippius & Neundörfer, 2003). Cognitive processes are first disrupted by Aβ oligomers in the synapse, and later by the formation of extracellular Aβ plaques around neurons (LaFerla et al., 2007). Aβ plaques develop from the aggregation of Aβ, a small protein (4 kDa), that develops from the amyloidogenic cleavage process of the membrane protein, amyloid precursor protein (APP) (LaFerla et al., 2007; LaFerla & Oddo, 2005).

In the healthy brain, APP is cleaved by the enzyme alpha secretase (α-secretase), and subsequently cleaved by the enzyme gamma secretase (γ-secretase), resulting in the formation of the protein known as P3 (LaFerla et al., 2007; LaFerla & Oddo, 2005). Conversely, when APP is cleaved under pathogenic conditions, via the amyloidogenic pathway, it is first cleaved by the enzyme beta secretase (BACE1), and then cleaved by γ-secretase (LaFerla et al., 2007; LaFerla & Oddo, 2005). This amyloidogenic cleavage process results in the formation of an Aβ peptide (LaFerla & Oddo, 2005). See Figure 1.

Aβ is originally produced as a monomer, yet it has the potential to combine with other Aβ peptides to form dimers, trimers, and fibrils. The aggregation of $A\beta_{1–40}$and $A\beta_{1–42}$ peptides results in the formation of insoluble Aβ plaques in the brain (LaFerla & Oddo, 2005). Specifically, $A\beta_{1–42}$ is 42 amino acid residues in length and more hydrophobic, thus making it more susceptible to insoluble Aβ plaque formation. Accordingly, Aβ plaques impede normal synaptic function and injure dendrites and axons (P. P. Liu et al., 2019). Although Aβ plaques are a hallmark pathology of AD, Aβ dimers, trimers, and oligomers are, themselves, also considered pathogenic (LaFerla & Oddo, 2005; Seixas da Silva et al., 2017).

An additional hallmark pathology of AD is hyperphosphorylated neurofibrillary tau tangles (LaFerla & Oddo, 2005). In the healthy brain, tau is a microtubule-associated protein

(MAP) that facilitates microtubule formation and axoplasmic transport (Gong & Iqbal, 2008). Microtubules are essential for proper neuronal function, as they help form the neuron's cytoskeleton, and regulate cell signaling, communication, and intracellular trafficking. Conversely, in a disease state, tau is hyperphosphorylated, resulting in the formation of filaments known as neurofibrillary tangles (Gong & Iqbal, 2008). In the AD brain, hyperphosphorylated tau tangles disrupt fundamental cellular processes, like microtubule production and axoplasmic transport in neurons (Gong & Iqbal, 2008). Consequently, the extracellular aggregation of Aβ, in addition to the intracellular formation of hyperphosphorylated neurofibrillary tau tangles, impairs cellular functions and ultimately results in neuronal death (LaFerla & Oddo, 2005).

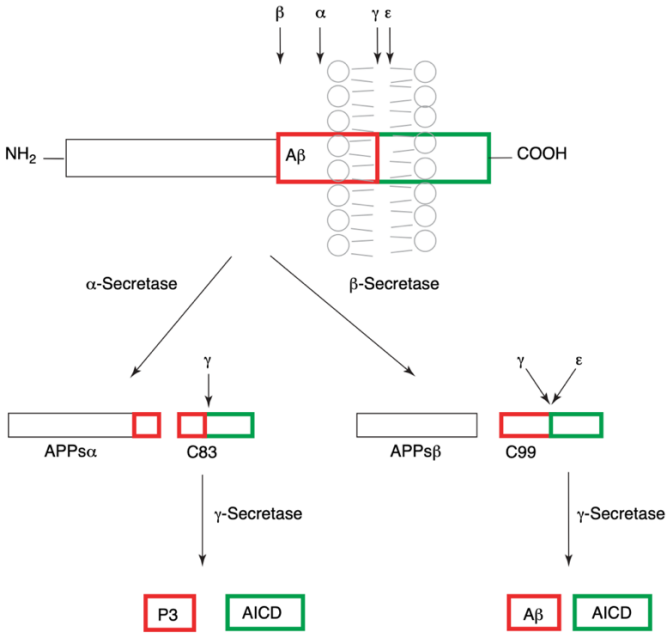


Figure 1. Aβ is generated via the amyloidogenic cleavage of APP (LaFerla & Oddo, 2005)

In addition to his discovery of hallmark AD pathologies, Dr. Alzheimer also noted that AD engenders severe brain atrophy, or shrinkage of the brain (Hippius & Neundörfer, 2003). AD patients lose a significant amount of brain volume, and prior research has demonstrated that AD pathologies specifically provoke hippocampal atrophy (Bobinski et al., 2000; Gosche et al.,

2002). For example, Gosche et al. (2002) measured hippocampal volume in elderly nuns from the Mankato nun study and found that dementia patients had a smaller hippocampus in comparison to healthy individuals.

AD provokes an extensive loss of synapses throughout the brain, in addition to cholinergic neuronal death in the basal forebrain (Pepeu & Grazia Giovannini, 2017; Wu et al., 2005). The brain's cholinergic system plays a fundamental role in facilitating learning and memories processes, and cholinergic neurons in the basal forebrain project to the cerebral cortex and hippocampus (Wu et al., 2005).Thus, researchers hypothesize that the death of cholinergic neurons could be implicated in the development of AD pathologies. This cholinergic hypothesis led to the development of AD therapeutics that target the brain's cholinergic system, such as acetylcholinesterase inhibitors (Bekris et al., 2010; P. P. Liu et al., 2019). These drugs prevent the enzymatic degradation of acetylcholine, and have previously been used as an AD therapeutic (P. P. Liu et al., 2019). However, acetylcholinesterase inhibitors only temporarily relieve AD symptoms, and do not prevent further advancement of disease pathologies. Hence, researchers need to find more efficient AD therapeutics that not only ease symptoms, but also provide long-term treatment to AD patients.

There are two subtypes of AD, including familial AD (FAD) and sporadic AD (SAD). FAD, also known as early onset AD, only accounts for about 6% of all AD cases and it is typically diagnosed before the age of 65 ("2021 Alzheimer's disease facts and figures," 2021). Scientists have already discovered three gene mutations linked to the generation of FAD, including APP, presenilin-1, and presenilin-2, all of which are implicated in amyloidogenesis (LaFerla et al., 2007; LaFerla & Oddo, 2005). Conversely, SAD, also known as late onset AD, accounts for the about 94% of all AD cases and commonly develops after the age of 65 ("2021

Alzheimer's disease facts and figures," 2021). The etiology of this more common form of AD is still largely unknown. However, recent research has revealed that there are gene mutations associated with SAD, including apolipoprotein E (APOE) and Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) ("2021 Alzheimer's disease facts and figures," 2021). APOE is typically expressed in astrocytes and assists cells in lipid transport and lipoprotein production (Shi & Holtzman, 2018). More specifically, the $APOE_4$ allele has been shown to increase the toxicity of Aβ and further exacerbate the immune response (Shi & Holtzman, 2018), and homozygous $APOE_4$ is consistently associated with increased AD risk (Vlachos et al., 2021).

Additionally, the TREM2 mutation, R47H, has been shown to increase one's risk of developing AD. TREM2 is typically expressed in microglial cells, the brain's resident phagocyte, and the R47H mutation disrupts proper microglia function and increases susceptibility to injury and infection in the brain (Shi & Holtzman, 2018). In addition to these mutations, several SAD risk factors have been identified, and need to be further investigated to find more effective prevention or intervention strategies.

1b. AD Pathologies and Inflammation

Collectively, our laboratory studies different environmental risk factors, such as chronic stress or poor diet, that may induce an inflammatory state and provoke the development and progression of AD pathologies. Notably, prior research has repeatedly demonstrated that there is a clear link between inflammation and AD neuropathology (Bonaiuto et al., 1997; Liu et al., 2012; Newcombe et al., 2018). Inflammation occurs primarily due to activation of the innate immune system, and is an adaptive response utilized to protect the body from unknown inflammatory triggers, such as pathogens, allergies, pollutants, and unhealthy food (Macdonald & Monteleone, 2005; Parham, 2013). These inflammatory triggers activate the innate immune

response, and immune cells and other cells are recruited to secrete pro-inflammatory cytokines (Dantzer, 2018; Dantzer & Kelley, 2007; Parham, 2013).

Cytokines are small proteins that are released to induce inflammation at sites of injury or infection throughout the body (Parham, 2013). Initially, this is an adaptive process that protects the body and hinders infection (Dantzer & Kelley, 2007; Maier & Watkins, 1998). For example, Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine that plays a crucial role in the innate immune response, and prior research has also shown that low levels of IL-1β are necessary during learning and memory processes (Takemiya et al., 2017). Although cytokine production is initially advantageous, chronic or excessive pro-inflammatory cytokine production engenders a constant inflammatory state that is harmful to the brain (Block et al., 2007).

Although scientists originally described the brain as an immune-privileged site, largely devoid of immune activity, recent research has demonstrated otherwise. As previously discussed, immune cells, such as macrophages, induce inflammation in injured or infected areas throughout the periphery of the body (Parham, 2015). Likewise, in the CNS, microglial cells are the resident phagocyte, and function like immune cells to protect the brain (Lenz & Nelson, 2018). Cytokines that are synthesized in their periphery of the body may enter the CNS via active and passive transport, and other mechanisms, such as circumventricular organs (CVOs) (Dantzer et al., 2008; Goshen & Yirmiya, 2009). Further, vagal afferents sense cytokine production in the periphery of the body and induce de novo production of cytokines in the brain, typically via microglial cells (Biesmans et al., 2013; Dantzer, 2018; Steinberg et al., 2016; Zanos et al., 2018). Microglia have many important tasks, such as providing structural support to neurons and performing phagocytosis in response to unfamiliar pathogens (Block et al., 2007). Initially, microglial cell activation is an advantageous and protective mechanism in the CNS. However, in a disease state,

like AD, microglia may become chronically activated and adopt a pro-inflammatory phenotype (Block et al., 2007). Under these conditions, microglial cells continually secrete pro-inflammatory cytokines and other neurotoxic factors, such as reactive oxygen species, that are harmful to neurons (Block et al., 2007; Lenz & Nelson, 2018). For example, continual secretion of neurotoxic factors makes the brain more susceptible to oxidative stress. The brain's oxidative system requires a proper balance of pro-oxidants and antioxidants, and excess reactive oxygen species disrupt this system, and induce oxidative stress in the brain (Gandhi & Abramov, 2012). Oxidative stress is interconnected with AD neuropathology, inflammation, and microglial cell activation. Hence, microglia's once-protective role may become destructive to neurons and other brain cells. This self-perpetuating, cyclical process, known as microgliosis, further exacerbates inflammation in the CNS, and leads to neuronal death (Block et al., 2007). See Figure 2.

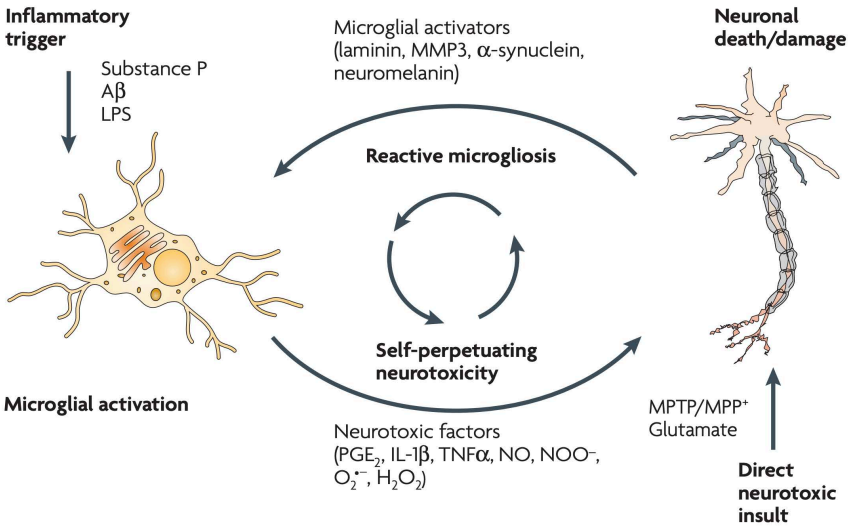


Figure 2. Microgliosis results in neuronal death (Block et al., 2007).

Preceding research has clearly demonstrated that pro-inflammatory mediators are connected to AD pathologies (Dursun et al., 2015; Engelhart et al., 2004; Gezen-Ak et al., 2013). For example, Dursun and colleagues (2015) found that early onset AD patients have significantly

increased levels of IL-1β and IL-6 in their serum compared to healthy individuals. Further, Engelhart et al. (2004) reported that middle-aged adults with higher levels of pro-inflammatory mediators, including TNF-α, IL-6, IL-1β, and C-reactive protein (CRP), have an increased risk of developing AD. Collectively, this research suggests that increased pro-inflammatory cytokine production in the periphery of the body may provoke or exacerbate AD pathogenesis in the CNS.

In support of this hypothesis, our laboratory has previously demonstrated that repeated exposure to inflammatory stimuli generates hippocampal $A\beta_{1-42}$ and cognitive dysfunction in wildtype mice that are not predisposed to AD (Kahn et al., 2012; Kranjac et al., 2012; Weintraub et al., 2013; Weintraub et al., 2014). Our laboratory works with wildtype mice to study SAD, as the etiology of SAD is still in question, and remains the more prevalent subtype of AD. Previous work has shown that lipopolysaccharide (LPS), an element from the cell wall of gram-negative bacteria, induces an inflammatory response in wildtype mice (Kahn et al., 2012; Lee et al., 2008). During the innate immune response, pattern recognition receptors (PRRs) on immune cells detect pathogen-associated molecular patterns (PAMPS) that are present on pathogen surfaces, and thus activate immune signaling pathways (Dantzer, 2009; Parham, 2013). Upon activation, immune cells secrete pro-inflammatory mediators, such as type I interferon, to protect the body.

Toll-like receptors (TLRs) are PRRs that play a key role during the immune response (Kawasaki & Kawai, 2014). For example, when PAMPS are detected, toll-like receptor 4 (TLR4) recruits the protein MyD88 and initiates its movement to the cell surface. Next, MyD88 activates signal transduction pathways, and subsequently activates the transcription factor NF-κB, which, in turn, facilitates the transcription of pro-inflammatory cytokines (Kawasaki & Kawai, 2014; Lee et al., 2003). LPS is a TLR4 agonist that initiates this process, and ultimately

induces pro-inflammatory cytokine secretion (Kawasaki & Kawai, 2014). Historically, our laboratory has demonstrated that seven consecutive days of intraperitoneal LPS injections induces cognitive dysfunction and the production of soluble Aβ in the hippocampus of wildtype, C57BL/6 mice that are not genetically predisposed to AD (Kahn et al., 2012; Weintraub et al., 2013).

Additionally, we study different environmental factors that may further exacerbate LPS-induced inflammation, to explore potential causal factors involved in SAD in wildtype mice, as they do not generate large amounts of soluble Aβ without an inflammatory stimulus present. Furthermore, our laboratory has also found that repeated exposure to a viral mimetic and TLR3 agonist, poly I:C, disrupts contextual fear memory consolidation and engenders soluble hippocampal Aβ production in C57BL/6 mice (Weintraub et al., 2014). Collectively, these data suggest that repeated inflammatory exposure may contribute to the development of SAD, and thus, the implementation of anti-inflammatory strategies could be key for AD therapeutics.

1c. AD Risk Factors

Prior research has identified several environmental risk factors for AD, such as aging, chronic stress, poor cardiovascular health, diabetes mellitus type II, and sedimentary lifestyle ("2023 Alzheimer's disease facts and figures," 2023; Baranowski, Hayward, et al., 2018; Livingston et al., 2020). Most recently, mid-life obesity has been reported as the number one modifiable risk factor for AD (Livingston et al., 2020). Like AD, obesity prevalence has increased at an alarmingly high rate in the United States over the last few decades. The CDC reported that over 72% of Americans are overweight or obese (Fryar et al., 2021; Fryar CD, 2020; Hales et al., 2018). In 2016 alone, Americans spent nearly $480 billion dollars on healthcare and treatment for chronic diseases that are typically comorbid with obesity (Burguera

et al., 2021). Notably, healthcare for obesity-induced chronic diseases accounts for almost half of the total costs for chronic disease treatment in the U.S (Waters, 2018).

Obesity prevalence has most likely increased due to the introduction of novel dietary factors in the United States (Cordain et al., 2005; Simopoulos, 2006). The American diet has rapidly evolved over the last few decades, and Americans are choosing more convenient, time- and cost-efficient meal choices (Simopoulos, 2006). Novel dietary components, such as refined grains, sugars, chemicals, dyes, pesticides, and preservatives, have been introduced to Americans throughout the last century (Cordain et al., 2005; Simopoulos, 2006). The typical American diet, notoriously known as the Western diet (WD), contains bountiful processed ingredients that may lead to severe health consequences, including obesity, which are commonly comorbid with several other diseases, such as cardiovascular disease and diabetes mellitus type II (Barnard et al., 2014; Bruce-Keller et al., 2009; Mozaffarian et al., 2010; Whitmer et al., 2007). Furthermore, prior research has demonstrated that increased body weight and body mass index (BMI) are correlated with an increased risk of developing AD, and individuals with a BMI over 25 kg/m^2 are typically considered overweight or obese (Naderali et al., 2009; Raatz et al., 2017). Specifically, mid-life obesity further increases one's risk of developing AD, especially in women (Whitmer et al., 2007).

2. Introduction to the Western Diet

Cordain and colleagues studied Western dietary patterns and found that 72.1% of the total energy consumed by Americans is composed of refined cereals, sugars, and vegetable oils, in addition to high-fat dairy products, and alcohol (Cordain et al., 2005). See Figure 3. Additionally, the WD is notorious for its poor nutritional value, as it is commonly filled with processed foods that lack vital nutrients and fiber. Fruits and vegetables, along with other

complex carbohydrates, are key sources of fiber that are essential for human health. Notably, only a paltry 10% of Americans consume the recommended servings of fruits and vegetables per day (Lee et al., 2022).

An additional contributor to America's rise in obesity and chronic disease is fast-food (Fryar, 2018; Simopoulos, 2006; Urban et al., 2014). Urban and colleagues (2014) found that fast-food consumption has rapidly increased within the last thirty years, and Americans consumed about 83% more fast food in 2010 in comparison to the late 1900's. More recent data have also revealed that 1 out of 3 Americans consume fast food daily (Fryar, 2018). Overall, this substantial increase in fast-food consumption is likely perilous to human health. For example, Urban and colleagues (2014) found that a large-sized combo meal at a fast-food restaurant, including a cheeseburger, French fries, and soda usually provides about 65 – 80% of calories in a 2,000-calorie per day diet. Thus, Americans are consuming copious amounts of animal-based fat and protein, as well as refined carbohydrates and sugars, and evidence suggests that increased consumption of these four dietary factors may increase one's risk of developing AD.

Food and food types found in Western diets generally una preagricultural hominins[1]

Food or food group
Dairy products
Whole milk
Low-fat milk
Cheese
Butter
Other
Total
Cereal grains
Whole grains
Refined grains
Total
Refined sugars
Sucrose
High-fructose corn syrup
Glucose
Syrups
Other
Total
Refined vegetable oils
Salad, cooking oils
Shortening
Margarine
Total
Alcohol
Total energy
Added salt, as sodium chloride

[1] Data adapted from references 22–24.
[2] In the US diet.
[3] Salt from processed foods, table salt use, and cook

Figure 3. Dietary Factors and Total Energy Consumed by Americans (Cordain et al., 2005). Cordain and colleagues (2005) reported that about 48.2% of the typical American diet is comprised of refined vegetable oils, sugar, high-fat dairy, and alcoholic beverages.

2a. Fatty Acids in the Western Diet

The average total fat intake for an American adult is approximately 34% kilocalories (kcal) from fat, specifically 33.6% kcal from fat, for men, and 33.5% kcal from fat, for women (Wright & Wang, 2010). There are four different types of fatty acids, including saturated fatty acids (SFA), trans-unsaturated fatty acids, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). The WD is largely composed of SFA, which is commonly found in animal-based foods, such as processed meat and high-fat dairy products, and in some plant-based foods, such as coconut oil and palm oil (Huth et al., 2013). While most SFA's in the WD are found in animal-based foods, like red meat, milk, cream, butter, and cheese, unsaturated fatty acids typically come from plants, such as oils, nuts, and legumes. Shan and colleagues

(2019) examined dietary trends and macronutrient intake among American adults from 1999 to 2016 and found that SFA consumption has increased by 36% over the last two decades (Shan et al., 2019). Data from this study also revealed that American adults typically consume about 12% kcal from SFA per day, even though it is recommended to consume less than 10% kcal from SFA per day (Shan et al., 2019). While some fatty acids are nutritionally advantageous, like MUFAS, large quantities of SFA can be detrimental to one's health. There are many ramifications associated with consuming large quantities of SFA, such as increased LDL cholesterol in the blood, that may increase one's risk of having heart disease (Mozaffarian et al., 2010).

2b. The Harmful Effects of Western Diet Fatty Acids

Additionally, previous research has also indicated that increased consumption of SFA (Kien et al., 2015; Poppitt et al., 2008; van Dijk et al., 2009) and red meat (Montonen et al., 2013) is associated with increased pro-inflammatory mediators in adults. For example, a previous study found that a Western-style diet, rich in SFA, increased inflammatory gene expression in the adipose tissue of adults at risk of developing metabolic syndrome (van Dijk et al., 2009). Accordingly, research in human subjects has shown that the inflammatory nature of high SFA diets increases one's risk of developing several diseases, such as heart disease (Mozaffarian et al., 2010) and dementia (Kalmijn et al., 1997).

Compared to other dietary patterns around the world, the WD contains more SFA in comparison to unsaturated fatty acids. As previously stated, there are two types of unsaturated fatty acids, including MUFAS and PUFAS, and there are two additional subtypes of PUFAS, including omega-6 and omega-3 fatty acids (Bazinet & Layé, 2014). Although PUFAS are necessary for proper brain development and cognitive function, nutritional research has

demonstrated that a balanced ratio of PUFAS is imperative to human health. The recommended ratio of omega-6 to omega-3 is 1:1 or 2:1, however, the average American typically consumes an omega-6 to omega-3 ratio of 15:1(Cordain et al., 2005; Simopoulos, 2006). Refined oils, such as safflower oil, corn oil, soybean oil, palm oil, and coconut oil, are the key omega-6 sources in the Western diet. Although small amounts of omega-6 are healthy and even considered anti-inflammatory, a proper balance of omega-6 to omega-3 is crucial in disease prevention (Simopoulos, 2016).

Furthermore, the WD lacks essential omega-3 fatty acids, and omega-3 deficiency is associated with an increased risk of developing obesity and cognitive decline (Bazinet & Layé, 2014; Phillips et al., 2012). Prior research has also revealed that AD patients have significantly lower levels of a subtype of omega-3 fatty acid, called docosahexaenoic acid (DHA), in comparison to healthy adults (Prasad et al., 1998; Söderberg et al., 1991). Overall, previous studies suggest that omega-3 fatty acid deficiency, in addition to substantial omega-6 and SFA intake, may act as a significant risk factor for AD.

2c. Protein in the Western Diet

The WD contains plentiful animal-based proteins rather than plant-based proteins. The CDC reported that men typically consume about 16% kcal from protein, while women consume about 15.5% kcal from protein per day (Wright & Wang, 2010). Animal-based protein intake has significantly increased in America since the year 2000 (Wright & Wang, 2010). Typical protein sources in the WD include a variety of animal-based proteins, such as processed meat, high-fat dairy products, casein protein, and eggs. Collectively, prior research has also revealed that increased consumption of animal-based protein is correlated with AD development (Dodge et al., 2012; Grant, 2014).

2d. Refined Carbohydrates and Fiber Deficiency in the Western Diet

The WD is largely comprised of refined carbohydrates, grains, and sugars that are typically found in foods like white bread, baked goods, and pastries. The typical WD provides about 50% kcal from carbohydrates per day (Wright & Wang, 2010). However, it contains superfluous amounts of simple carbohydrates in comparison to fiber-rich, complex carbohydrates (Cordain et al., 2005). For example, Cordain and colleagues (2005) found that Americans typically consume 24% kcal from cereal grains per day, and refined grains make up 20.4% kcal of total energy, while whole grains only make up 3.5% kcal of total energy per day. Fiber is essential for both gut and brain health, due to its ability to regulate blood sugar and aid in digestion. Although nutritionists recommend that people should consume at least 14 grams of fiber per 1,000 kcal consumed per day, a recent study reported that only about 7.4% of American adults meet this recommendation (Miketinas et al., 2021). Thus, the average American diet is vastly fiber deficient, and this could render serious health consequences.

There are two key types of fiber, including both soluble and insoluble fiber. First, soluble fiber plays an important role in the regulation of blood sugar and cholesterol (Brown et al., 1999). Therefore, soluble fiber consumption is crucial for disease prevention, such as diabetes mellitus type II, which is a known risk factor for AD. Indeed, prior studies have already demonstrated that soluble fiber, like inulin, reduces inflammation in diabetes mellitus type II patients (Dehghan, Gargari, et al., 2014; Dehghan, Pourghassem Gargari, & Asghari Jafar-abadi, 2014). Typical sources of soluble fiber include plant-based foods, such as oatmeal, nuts, legumes, beans, and fruit. Second, insoluble fiber, commonly found in foods such as wheat products, brown rice, and leafy greens, is also necessary for proper digestion (Guan et al., 2021).

2e. Fiber Deficiency and the Gut Microbiome

In addition to slowing down digestion and preventing a spike in blood glucose levels, a high-fiber diet is necessary to maintain essential bacteria in the gut microbiome (Turnbaugh et al., 2009). For example, a fiber-rich diet provides plentiful microbiota-accessible carbohydrates (MACs) that lead to the production of vital short-chain fatty acids, like butyrate, in the intestines and colon, and, therefore, stimulates growth of essential and healthy microbiota (Sonnenburg & Sonnenburg, 2014). However, the WD does not provide enough MACs to maintain gut essential and diverse microbiota (Desai et al., 2016; Sonnenburg & Sonnenburg, 2014).

Previous research has demonstrated that fiber-deficient diets, such as the WD, may increase one's risk of developing chronic diseases, like diabetes mellitus type II (McKeown et al., 2004; Schulze et al., 2004; Weickert & Pfeiffer, 2018). The gut and brain are connected via the gut-brain axis, and essential microbiota are known to influence human health and behavior through multiple interactive mechanisms (Burokas et al., 2015). For example, the presence of essential gut bacteria, such as *Bifidobacterium*, has been associated with reduced inflammation in the gut, whereas gram-negative bacteria and other harmful microbes have been shown to exacerbate inflammation in the gut of C57BL/6 mice (Li et al., 2018). Indeed, as the gut and brain are functionally interconnected, it is important to maintain a healthy gut microbiome and prevent excess inflammation, as this may influence brain health, as well (Burokas et al., 2015).

2f. Refined Sugars in the Western Diet

Refined carbohydrates and sugars are considered high glycemic load foods, because they increase blood glucose, insulin resistance, adiposity, and body weight, all of which are known risk factors for diabetes mellitus type II and AD (Christ et al., 2019; Cordain et al., 2005; Elliott et al., 2002; Hill et al., 2019; McKeown et al., 2004; Schulze et al., 2004). Collectively, prior research has revealed that high glycemic index foods, like refined cereal grains, make up almost

40% of the total energy provided by the (Cordain et al., 2005). Therefore, these data indicate that Americans are not consuming enough whole grains, and they are choosing less nutrient dense, high glycemic carbohydrate sources. Additionally, refined sugars provide 19% of the total energy from the typical WD (Cordain et al., 2005).

As previously stated, about 90% of Americans do not consume the recommended number of fruits and vegetables (Lee et al., 2022), so most sugar in the WD is obtained through the consumption of high glycemic load foods, like white bread, refined cereals, pastries, and desserts (Cordain et al., 2005). The average American consumes about 13% kcal from refined sugars per day, even though nutritionists recommend consuming less than 10% kcal from sugar per day (*Nutrient intakes from food and beverages: mean amounts consumed per individual, by gender and age, What We Eat in America, NHANES 2013–2014.* , 2016; Urban et al., 2014). Furthermore, another study reported about 18.6% of total energy in the WD was provided by refined sugars (Cordain et al., 2005).

Consequently, research in senior adults has shown that excessive consumption of refined grains and sugars increases AD biomarkers (Taylor et al., 2017). Taylor and colleagues (2017) found that increased consumption of refined grains and sugars induces Aβ production in the cortical region of cognitively healthy, senior adults. Additionally, seniors who consumed excess refined grains and sugars received lower scores on cognition tests in comparison to seniors who consumed a healthier diet (Taylor et al., 2017). Overall, the WD has been associated with increased disease risk, and thus, the implementation of healthier diets in the U.S. could target modifiable risk factors of AD and potentially reduce AD prevalence (Livingston et al., 2020). Emerging research suggests that the Mediterranean diet (MD) could be utilized to target AD risk factors, as it has already been associated with reduced risk of mortality (Trichopoulou et al.,

2003; Trichopoulou et al., 1995; Trichopoulou & Vasilopoulou, 2000), weight gain and obesity (Poulimeneas et al., 2020; Sánchez-Villegas et al., 2006; Schröder et al., 2004), and 34% less risk of AD (Gu et al., 2010) in older adults.

3. Introduction to the Mediterranean Diet

In a few areas of the world, AD prevalence is minimal, and almost nonexistent. These areas are known as Blue Zones, and they are home to the world's largest populations of centenarians (Buettner, 2015). National Geographic journalist and researcher, Dan Buettner, identified five Blue Zones around the world, including Loma Linda (California), Nicoya (Costa Rica), Sardinia (Italy), Icaria (Greece), and Okinawa (Japan). People who reside in Blue Zones are known for their exceptional dietary patterns, active lifestyles, and longevity. Notably, two Blue Zones, including Sardinia and Ikaria, border the Mediterranean Sea, and increased longevity and low disease prevalence in these regions is largely attributed to adherence to the traditional MD (Buettner, 2015; Chrysohoou et al., 2004; Legrand et al., 2021; Panagiotakos et al., 2011; Trichopoulou, 2004; Trichopoulou et al., 2003; Trichopoulou & Vasilopoulou, 2000).

The MD is the typical dietary pattern among people who live in olive-growing regions bordering the Mediterranean Sea (Trichopoulou, 2004; Trichopoulou et al., 2003; Trichopoulou et al., 2014). Key components of the MD include high consumption of monounsaturated fatty acids (MUFAS), the main fat source in olive oil, legumes, complex carbohydrates, fruits, vegetables, and moderate consumption of red wine, milk and low-fat dairy products (Bach-Faig et al., 2011; Trichopoulou, 2004). Notably, the MD is a mainly plant-based, although poultry, fish, and other meat products are still consumed sparingly (Trichopoulou, 2004). People who strictly adhere to the MD typically eat fruits, vegetables, complex carbohydrates, and olive oil with every meal (Bach-Faig et al., 2011). Additionally, they typically consume two servings of

low-fat dairy, and two servings of olives or nuts each day. Fish, poultry, and eggs are also consumed about two times per week, while red meat is typically consumed three to four times per month, see Figure 5 (Bach-Faig et al., 2011).

Trichopoulou and colleagues (1995) studied the potential health benefits of the MD in Greek adults and found that lifelong adherence to the traditional MD significantly reduced mortality risk by 17%, and fruits and vegetables were the most important components to protect against mortality. Dr. Antonia Trichopoulou was the first researcher to create the Mediterranean-diet score, a scale that accurately depicts adherence to the traditional MD (Trichopoulou et al., 2003; Trichopoulou et al., 1995). The Mediterranean Diet Score measures MD adherence by scoring the consumption of nine dietary factors, including vegetables, legumes, fruits and nuts, fish, dairy products, cereals, meat and poultry, and alcohol (Bach-Faig et al., 2011; Trichopoulou et al., 2003; Trichopoulou et al., 1995). A high MD score (maximum score of 9) signifies strict adherence to the traditional MD (Bach-Faig et al., 2011; Trichopoulou et al., 2003; Trichopoulou et al., 1995), and an increased score of 2 points on the Mediterranean-diet scale was associated with a 25% reduced risk of total mortality (Trichopoulou et al., 2003).

Figure 4. The Mediterranean Diet Pyramid (Bach-Faig et al., 2011).

Several studies have utilized magnetic resonance imaging (MRI) to investigate the effects of the MD on the brain, and close adherence to the MD has been associated with protection from AD pathologies, including amyloid beta (Aβ) (Berti et al., 2018; Rainey-Smith et al., 2018), brain atrophy (Gu et al., 2015; Kaplan et al., 2022; Mosconi et al., 2014; Staubo et al., 2017), and cognitive decline (Féart et al., 2009; Qin et al., 2015; Scarmeas et al., 2009; Tangney et al., 2011; Trichopoulou et al., 2015; Tsivgoulis et al., 2013) in middle-aged and elderly adults. Specifically, high adherence to the MD has been associated with reduced ventricular enlargement and atrophy in the hippocampus and temporal cortex of middle-aged adults (Gu et al., 2015; Mosconi et al., 2014), while low adherence has been shown increase atrophy and induce cortical thinning (Mosconi et al., 2014). A recent study provided even more evidence for the neuroprotective benefits of the MD, and reported that the MD was associated with reduced global AD pathology and Aβ in post-mortem brains of elderly adults from the Rush Memory and Aging Project. Among each MD component, high leafy green intake was associated with the lowest global AD pathology (Agarwal et al., 2023)

3a. Fatty Acids in the Mediterranean Diet

There are several variations of the MD, such as Italian, Greek, and Israeli, and each variant of the MD provides different amounts of macronutrients and micronutrients. For example, Greeks typically consume about 40% kcal from fat, while Italians consume about 30% kcal from fat (Trichopoulou et al., 2003; Trichopoulou et al., 2014). Overall, research in human subjects has established that the typical MED provides about 35– 40% kcal from fat (Trichopoulou, 2004; Trichopoulou et al., 2003; Trichopoulou et al., 2014). The main fat source in the MD is olive oil, as it is commonly consumed with every meal (Bach-Faig et al., 2011). For

example, in Ikaria, olive oil provides more than half of the total energy from fat per day (Buettner, 2015; Legrand et al., 2021).

The MD is considered a high-fat diet, due to abundant consumption of unsaturated fatty acids. Indeed, MUFAS and PUFAS, are thought to be potent contributors to the protective benefits of the MD (Trichopoulou et al., 2003). For example, Trichopoulou and colleagues (2003) found that increased consumption of MUFAS, in comparison to SFA, is associated with a decreased risk of mortality. Omega-9 is a type of MUFA that is a key dietary factor in the MD, and it is typically provided through olive oil consumption (Bach-Faig et al., 2011; Panagiotakos et al., 2011). Omega-9, such as oleic acid, is essential to brain health, and it is involved in several cellular processes, including myelin sheath formation (Bazinet & Layé, 2014). Moreover, a review reported that the typical MD provides about 19% kcal from MUFAS and about 5% kcal from PUFAS (Davis et al., 2015), while other researchers have reported that the typical Greek MD provides about 3.6% kcal from PUFAS (Trichopoulou et al., 2005).

3b. The Neuroprotective Role of Fatty Acids in the Mediterranean Diet

Omega-6 and omega-3 long-chain fatty acids are an important component of the MD, and are commonly obtained through olive oil, nuts, and fatty fishes (Simopoulos, 2001). As previously discussed, it is crucial to consume a balanced ratio of omega-6 to omega-3, and the MD typically provides a ratio of 1:1 or 2:1 (Davis et al., 2015; Simopoulos, 2001; Trichopoulou et al., 2005). Omega-6 and omega-3 fatty acids are imperative for brain development, learning, and memory, as they play an important role in neuronal membrane structure and aid in important cellular activities (Bazinet & Layé, 2014; Cutuli, 2017; Dyall, 2015). For example, PUFAS are broken down by enzymes, and their mediators assist in the regulation of neuroinflammation, synaptogenesis, and synaptic plasticity (Bazinet & Layé, 2014).

Notably, 35% of the total fatty acids in the human brain consist of omega-6 and omega-3 fatty acids (Bazinet & Layé, 2014; Luchtman & Song, 2013). However, PUFAS cannot be completely synthesized by the body alone, and must be attained through diet (Bazinet & Layé, 2014). Thus, diets containing a balanced ratio of omega-6 and omega-3 fatty acids, like the MD, could potentially provide neuroprotective benefits. There are two key subtypes of omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as a key subtype of omega-6, arachidonic acid (ARA). While other fatty acids, such as MUFAS and SFAs, can be created in the brain, PUFAS are typically created in the blood and can be transported to the brain via diffusion (Bazinet & Layé, 2014). Once fatty acids enter the brain, they are typically esterified into phospholipids, and DHA and ARA are the two most common fatty acids that make up the phospholipid membrane of cells. DHA is particularly important for synaptogenesis and cognition, as it has been shown to promote new hippocampal neurons *in vitro* (Calderon & Kim, 2004), and improve cognition in aging adults (Cutuli, 2017).

Due to DHA's ability to stimulate neurogenesis, it is a crucial dietary factor for proper brain development in infants (Innis, 2008). Prior research has demonstrated that the brain accumulates large amounts of DHA throughout gestation, as this is a key time in neural development, especially during the third trimester of pregnancy (Hadley et al., 2009; Kuipers et al., 2012). Additionally, prior studies have shown that DHA-rich baby formulas improve neurodevelopmental scores, in comparison to DHA deficient formulas, in young children (Cheatham et al., 2011; Lauritzen & Carlson, 2011). Notably, evidence suggests that DHA deficiency may be a contributing factor in AD pathogenesis, as a previous study reported that deceased AD patients had lower levels of DHA in the hippocampus compared to healthy controls (Lukiw et al., 2005).

Prior research has clearly established that combined fatty acids in the MD, largely provided through olive oil, fish, and nut consumption, facilitate protection against cognitive impairment (Martínez-Lapiscina et al., 2013; Scarmeas et al., 2009; Singh et al., 2014; Solfrizzi et al., 1999; Valls-Pedret et al., 2015), and inflammation (Casas et al., 2014; Casas et al., 2016; Casas et al., 2017) in elderly adults. For example, Casas and colleagues (2016, 2017) administered a MD, supplemented with olive oil or nuts, or a low-fat control diet, to older adults for five years, and found that both styles of the MD were protective against peripheral pro-inflammatory mediators. However, the MD enriched with high olive oil content was the most protective against inflammation, as it significantly decreased peripheral pro-inflammatory cytokine production in comparison to the other two diets (Casas et al., 2016; Casas et al., 2017). As inflammation is interconnected with AD pathologies, these data suggest that consistent consumption of an olive oil rich MD may protect elderly adults from diseases that have a chronic inflammatory component, such as AD.

3c. Protein Sources in the Mediterranean Diet

The MD typically provides about 15–20% kcal from protein (Trichopoulou et al., 2005). The average protein requirement for adults is about 41 grams per day, and the Greek MD provides about 74.5 grams of protein (Trichopoulou et al., 2005). People living in Mediterranean regions typically consume a variety of plant-based protein sources including beans, lentils, nuts, and legumes. Although the MD is largely plant-based, it still includes animal-based sources of protein, including eggs, poultry, fish, low-fat dairy products, such as yogurt, and long-preservable cheeses, that are consumed in minimal to moderate quantities (Bach-Faig et al., 2011; Buettner, 2015).

3d. Complex Carbohydrates and Fiber in the Mediterranean Diet

The primary macronutrient source in the MD is complex carbohydrates, and individuals living in Mediterranean regions typically consume about 40–48% kcal from carbohydrates per day (Davis et al., 2015; Trichopoulou et al., 2005). Key carbohydrate sources in the MD include whole grain bread, whole grain pasta, brown rice, legumes, and potatoes, although they vary by region around the Mediterranean Sea. For example, Ikarians consume high quantities of potatoes, while Sardinians consume large quantities of whole durum wheat and barley (Buettner, 2015). Durum wheat is high in fiber, complex carbohydrates, and protein. Additionally, barley bread is very popular in Sardinia, due to its high fiber content and low-glycemic index. Hence, fiber-rich breads and pastas made from ingredients like durum wheat or barely prevent a rapid spike in blood glucose following a meal (Buettner, 2015; Dehghan, Gargari, et al., 2014; Dehghan, Pourghassem Gargari, & Asghari Jafar-abadi, 2014). The MD provides about 3–5% kcal from dietary fiber per day (Trichopoulou et al., 2005). For example, the traditional Greek diet provides about 2.4% kcal from total fiber, roughly 29.8 grams. Soluble fiber, like inulin, is particularly beneficial because it has been shown to reduce peripheral inflammatory markers, like TNF-α and CRP, in diabetic, female patients (Dehghan, Gargari, et al., 2014; Dehghan, Pourghassem Gargari, & Asghari Jafar-abadi, 2014). The MD provides plentiful soluble fiber in berries, lentils, whole grain pasta, barley and nuts, whereas insoluble fiber is typically found in foods like brown rice, fruits, and vegetables (Buettner, 2015; Trichopoulou et al., 2005).

3e. High-Fiber Diets and the Gut Microbiome

Furthermore, fiber is essential for maintaining diverse microbiota and short-chain fatty acids in the gut (Statovci et al., 2017). The gut microbiome communicates with the brain largely via vagal afferents and the production of short-chain fatty acids, and, thus, has the potential to influence cognitive, emotive, and behavioral functions (Bourassa et al., 2016; Garcia-Mantrana

et al., 2018; García-Montero et al., 2021). More recent discoveries suggest that microbiome dysbiosis is largely associated with diseases, like obesity and diabetes mellitus type II (Garcia-Mantrana et al., 2018). As previously noted, high-fiber diets, like the MD, provide MACs, which subsequently induce the production of neuroprotective short-chain fatty acids, such as butyrate, acetate, and propionate (De Filippis et al., 2016; Garcia-Mantrana et al., 2018). For example, Garcia-Mantrana et al. (2018) found that healthy adults who closely adhered to the MD had increased microbial diversity and essential gut microbiota, such as *Catenibacterium,* and significantly higher levels of short-chain fatty acids, like butyrate. These findings suggest that the MD could potentially protect cognitive health via the gut-brain axis.

3f. Protective Antioxidants in the Mediterranean Diet

In addition to the previously discussed macronutrients, the MD provides plentiful plant polyphenols that are commonly found in olive oil, fruits, vegetables, tea, coffee, red wine, and Mediterranean herbs and spices. Traditional Mediterranean meals are cooked in a variety of wild herbs with anti-inflammatory properties, such as mint, rosemary, oregano, sage, and marjoram (Buettner, 2015). In addition to these anti-inflammatory properties, traditional Mediterranean dietary factors also provide antioxidants that are necessary for the brain's oxidative system. As previously discussed, the brain is susceptible to damage from reactive oxygen species (Gandhi & Abramov, 2012; Tönnies & Trushina, 2017), and natural antioxidants from plant-based foods could potentially be utilized to protect the brain from oxidative stress (Omar et al., 2017).

Plant polyphenols contain powerful antioxidant properties that combat oxidative stress and inflammation (Omar et al., 2017). For example, resveratrol is a plant polyphenol in the MD provided from berries, peanuts, grapes, and red wine. Prior studies have shown that resveratrol

supplementation activates the sirtuin 1 gene, a gene that is involved in neuronal plasticity, cognitive function, and mitochondrial activation (Borra et al., 2005). Additionally, olive oil provides antioxidants, and it contains over thirty phenolic compounds that have been shown to attenuate oxidative stress and inflammation *in vitro* and *in vivo* (reviewed by Abuznait et al., 2013). Furthermore, flavonoids are one of the most powerful plant polyphenols found in the MD. A review article reported that the mean flavonoid intake in the MD is about 344.9 milligrams per day, and high flavonoid intake may be a leading contributor to low disease prevalence in Mediterranean regions (Davis et al., 2015). Taken together, prior research has shown that multiple components of the MD may provide protection against AD biomarkers, inflammation, and cognitive impairment, and MD experts infer that it is the *synergy* of numerous dietary factors in the MD that provide protection against disease and mortality (Trichopoulou et al., 2015; Trichopoulou et al., 2014).

4. The Effects of the Western and Mediterranean Diets in Animal Research

In addition to research in human subjects, there is an overwhelming amount of evidence that key components of the WD provoke or exacerbate AD pathologies in both wildtype mice and transgenic mouse models of AD, respectively. Comprehensively, prior research has demonstrated that Western rodent diets induce obesity (Baranowski, Bott, & MacPherson, 2018; Guillemot-Legris, Mutemberezi, et al., 2016; C. Liu et al., 2019), behavioral alterations (Arnold et al., 2014; Gainey et al., 2016), cognitive impairment (Baranowski, Bott, & MacPherson, 2018; Boitard et al., 2014; Gabriel et al., 2020; Gainey et al., 2016; Heyward et al., 2012; Knight et al., 2014; Lin et al., 2016), microbial dysbiosis (Desai et al., 2016), and the onset or progression of inflammation (Guillemot-Legris, Masquelier, et al., 2016; Pistell et al., 2010), and AD

biomarkers (Busquets et al., 2017; Julien et al., 2010; Knight et al., 2014; Lin et al., 2016) in mice.

Conversely, prior studies have demonstrated that Mediterranean dietary factors incorporated into rodent diets are neuroprotective, although, there is a gap in the current scientific literature, as few studies have examined the effects of a *comprehensive* Mediterranean rodent on AD susceptibility in mice. For example, previous experiments have typically examined the effects of rodent diets supplemented with only a *single* Mediterranean dietary factor, rather than a comprehensive diet that mimics the typical human MD. Despite this clear limitation, numerous studies have demonstrated that individual components of the MD, like olive oil, fish oil, and plant polyphenols, are therapeutic against cognitive impairment (Farr et al., 2012; Lauretti et al., 2017; Petursdottir et al., 2008), AD biomarkers (Abuznait et al., 2013; Grossi et al., 2013; Lim et al., 2005; Sharman et al., 2019), inflammation (Fuccelli et al., 2018; Li et al., 2018), and microbial dysbiosis (Yu et al., 2019) in mice.

4a. The Effects of Diet on Body Weight in Rodents

A multitude of studies have demonstrated that a WD engenders excess body weight and obesity, in comparison to low-fat control diets, in mice (Arnold et al., 2014; Graham et al., 2016; Heyward et al., 2012; Lin et al., 2016; Pistell et al., 2010; Xu et al., 2018). For example, multiple studies have shown that SFA rich diets (40% kcal or 60% kcal from fat vs. a low-fat control diet) induce significant weight gain in male C57BL/6 mice (Pistell et al., 2010; Xu et al., 2018). However, other researchers have found that a WD does not significantly increase rodent body weight when it is compared to other high-fat diets. For example, Li and colleagues (2018) examined the effects of a Western rodent diet versus a plant-based, PUFA rich rodent diet, and failed to find significant differences in body weight in female C57BL/6 mice. Collectively, prior

research has confirmed that the Western diet increases body weight in comparison to low-fat rodent diets. However, more research is needed to explore the effects of high SFA Western diets in comparison to high unsaturated fat diets, like the MD.

As previously noted, there is limited research on the effects of a comprehensive MD in rodents. However, some studies have found that olive oil supplementation protects rodents from excess body weight compared to SFA rich diets (Lauretti et al., 2017; Nakajima et al., 2020). However, even when examining Mediterranean dietary factors, there are conflicting results in the scientific literature, as other studies have not found significant effects of such factors on body weight in wildtype mice and transgenic mouse models of AD (Marchlewicz et al., 2022; Nardiello et al., 2018; Sharman et al., 2019). More research is needed to understand the complex effects of different dietary patterns on body weight and obesity in rodents.

4b. The Effects of Diet on Cognition in Rodents

Prior research has clearly established that a WD disrupts learning and memory processes in rodents (Abbott et al., 2019; Kanoski & Davidson, 2011; Tsan et al., 2021). Western-style rodent diets have been shown to impair learning during hippocampus-dependent tasks, such as the Morris water maze and place recognition tasks, in both wildtype mice and transgenic mouse models of AD (reviewed in Abbott et al., 2019). For example, Western rodent diets, containing 40 – 60% kcal from fat (primarily SFA), have been shown to exacerbate or induce cognitive impairment in transgenic mouse models of AD (Kanoski et al., 2007; Knight et al., 2014; Lin et al., 2016; Walker et al., 2017), and male C57BL/6 mice (Heyward et al., 2012; Pistell et al., 2010; Xu et al., 2018).

On the other hand, rodent diets supplemented with Mediterranean dietary factors have been shown to improve cognition, and prior evidence suggests that PUFAS are a key dietary

factor that contribute to cognitive health. Notably, mice synthesize higher quantities of PUFAS in the blood rather than the brain, and the MD could potentially improve PUFA synthesis in rodents (Bazinet & Layé, 2014). For example, rodent diets supplemented with plant polyphenols, olive oil, or fish oil, have been shown to improve learning and memory in both male (Farr et al., 2012; Grossi et al., 2013; Nardiello et al., 2018) and female (Farr et al., 2012; Grossi et al., 2013; Lauretti et al., 2017; Li et al., 2018).

As noted stated, currently, only limited research exists addressing the effects of a complete MD on cognition in rodents. However, two recent studies examined the combined effects of multiple dietary components in the MD on cognition in mice (Li et al., 2018; Sharman et al., 2019). Sharman et al. (2019) administered diets containing several Mediterranean dietary factors, including curcumin, epigallocatechin-3-gallate, DHA and ALA fatty acids to a transgenic mouse model of AD. The results revealed that diets supplemented with curcumin and epigallocatechin-3-gallate increased hippocampus-dependent learning in contextual fear conditioning in transgenic mice (Sharman et al., 2019). Furthermore, Li and colleagues (2018) examined the effects of a PUFA-rich, plant-based diet versus a WD in female C57BL/6 mice. Mice were administered one of the two experimental diets for 3 months prior to behavioral testing, and the results revealed that the plant-based diet significantly enhanced spatial learning in comparison to a WD (Li et al., 2018). Moreover, research has also shown that short-chain fatty acids, produced from dietary fibers, are neuroprotective. For example, supplementation of sodium butyrate has been shown to improve learning and memory in rodents (Fernando et al., 2020; Govindarajan et al., 2011). As fiber is a key factor in the MD, more research is needed to examine the effects of diets rich in both soluble and insoluble fiber on learning and memory in rodents.

4c. The Effects of Diet on Behavior and Locomotor Activity in Rodents

In addition to influencing cognition, prior research has clearly established that a WD has increases anxiety-like behaviors, and decrease exploratory behaviors and locomotor activity in mice. For example, several studies have illustrated that Western rodent diets increase anxiety-like behaviors in both male (Gainey et al., 2016; Nakajima et al., 2020; Walker et al., 2017) and female rodents (Li et al., 2018; Walker et al., 2017). Additionally, prior studies have also demonstrated that Western rodent diets decrease exploratory behavior (Heyward et al., 2012; Knight et al., 2014; Oksman et al., 2006; Walker et al., 2017) and locomotor activity in both male (Gabriel et al., 2020; Nakajima et al., 2020; Oksman et al., 2006; Walker et al., 2017) and female rodents (Li et al., 2018; Walker et al., 2017).

On the contrary, Mediterranean dietary factor supplementation and plant-based diets have been shown to reduce anxiety-like behaviors and increase exploratory behaviors in male (Lauretti et al., 2017; Nakajima et al., 2020; Oksman et al., 2006) and female rodents (Li et al., 2018). Altogether, the previously discussed data suggest that Mediterranean dietary components may reduce anxiety and improve locomotor activity in male and female rodents compared to a WD.

4d. The Effects of Diet on Inflammation in Rodents

Prior research has also explored the relationship between the WD and inflammation in rodents, as pro-inflammatory cytokine production has been shown to influence behavior, learning, and memory processes (Dantzer & Kelley, 2007; Dantzer et al., 2008; Maier & Watkins, 1998). There is substantial evidence that both short-term and long-term consumption of a WD induces systemic inflammation in mice. Numerous studies have demonstrated that Western rodent diets increase peripheral pro-inflammatory cytokine production in wildtype mice

(Graham et al., 2016; Li et al., 2018; C. Liu et al., 2019; Pistell et al., 2010). For example, Pistell and colleagues (2010) and Li and colleagues (2018) both found that Western rodent diets significantly increased the production of TNF-α, IL-1β, and IL-6 in the serum of male and female C57BL/6 mice.

On the other hand, animal researchers have further studied the potential, anti-inflammatory effects of plant polyphenols found in olive oil, to understand olive oil's specific therapeutic properties. For example, several studies have examined the effects of hydroxytyrosol treatment on pro-inflammatory cytokine production in mice (Bitler et al., 2005; Fuccelli et al., 2018; Illesca et al., 2019; Rincón-Cervera et al., 2016). Furthermore, plant-based diets, supplemented with a variety of plant polyphenols, have also been shown to reduce peripheral pro-inflammatory mediators in mice. For example, Li and colleagues (2018) found that a plant-based diet significantly reduced TNF-α, IL-6, IL-1β, and MCP-1, and increased the anti-inflammatory cytokine, IL-10, in comparison to a WD. Likewise, fish oil or DHA supplementation has also been shown to lower peripheral pro-inflammatory mediators in mice (Akerele & Cheema, 2018; Xie et al., 2020).Although there is limited research on the potential, anti-inflammatory effects of a whole MD in rodents, these studies demonstrate that individual elements in the MD are anti-inflammatory. Hence, future research is needed to further investigate the potential, therapeutic capacity of a comprehensive MD on peripheral and central inflammation in rodents.

4e. The Effects of Diet on AD Pathologies in Rodents

In addition to promoting inflammation, plentiful studies have shown that Western rodent diets promote or exacerbate AD biomarkers, such as Aβ, in C57BL/6 mice (Busquets et al., 2017; Liu et al., 2014; Moser et al., 2018) and transgenic mouse models of AD (Graham et al.,

2016; Ho et al., 2004; Hooijmans et al., 2007; Julien et al., 2010; Oksman et al., 2006; Vandal et al., 2014; Walker et al., 2017), respectively. For example, there is substantial evidence that chronic consumption of Western rodent diets exacerbates hippocampal Aβ production and plaque deposition in the APP/PS1 mouse model of AD, in comparison to standard, control diets (Graham et al., 2016; Hooijams et al., 2007; Oksman et al., 2006). Additionally, both Julien et al. (2010) and Vandel et al. (2014) demonstrated that Western rodent diets exacerbate the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ in comparison to standard, control diets in the 3xTgAD mouse model of AD.

In contrast to the WD, individual components of the MD, like olive oil, have been shown to attenuate or prevent hippocampal and cortical $A\beta_{1-42}$ in transgenic mouse models of AD (Lauretti et al., 2017; Qosa et al., 2015) and wildtype mice (Abuznait et al., 2013), respectively. Additionally, previous studies have demonstrated that plant polyphenols in olive oil can be used as amyloid reducing agents in rodents (Grossi et al., 2013; Luccarini et al., 2014; Nardiello et al., 2018). For example, oleuropein aglycone, a plant polyphenol found in olive oil, attenuated Aβ plaques in a transgenic mouse model of AD (Grossi et al., 2013), and reduced $A\beta_{1-42}$ in Wistar rats treated with Aβ intracerebral injections (Luccarini et al., 2014). Hydroxytyrosol has also been shown to mitigate Aβ plaques in a mouse model of AD (Nardiello et al., 2018).

Similarly, fish oil and DHA (a primary long-chain fatty acid in fish oil) are also neuroprotective in rodents. Several studies have revealed that fish oil or DHA supplementation mitigates hippocampal and cortical Aβ production in transgenic mice (Green et al., 2007; Lim et al., 2005; Oksman et al., 2006; Perez et al., 2010; Zhou et al., 2018). For example, both Lim et al. (2005) and Perez et al. (2009) found that DHA supplemented rodent diets attenuated Aβ plaques in the hippocampus of transgenic mouse models of AD. Collectively, these data

demonstrate that Mediterranean dietary factors, particularly olive oil and fish oil, could potentially be utilized as amyloid lowering therapies.

4f. The Effects of Diet on the Liver-Brain Axis

More recent evidence suggests that impaired liver function, typically induced by nonalcoholic fatty liver disease (NAFLD), may potentially induce or exacerbate AD neuropathology in wildtype mice and transgenic mouse models of AD (Cheng et al., 2023; Kim et al., 2016). Notably, high SFA diets, like the WD, have been shown to engender NAFLD in mice (Castellanos-Tapia et al., 2020; Recena Aydos et al., 2019). The liver plays a pivotal role in the clearance of toxins, like peripheral Aβ, and thus is thought to be implicated in AD neuropathology. A damaged, fatty liver cannot properly clear Aβ from the blood, and thus, circulating Aβ can potentially transport to the brain via multiple mechanisms (Greenberg et al., 2020).

In a pathogenic state, like AD, the blood brain barrier (BBB) may be damaged, and abnormal transport of peripheral Aβ can occur through the receptor for advanced glycation end-products (RAGE receptor), which monitors peripheral Aβ entering the BBB (Greenberg et al., 2020; Wang et al., 2021). Although the complex cellular mechanisms involved in this process are not fully understood, a recent study found that the liver cleared about 13.9% of $A\beta_{1–42}$ from blood in the liver of APP/PS1 mice (Cheng et al., 2023). Further, the results of this studied also revealed that partial blockage of blood flow to the liver increased $A\beta_{1–42}$ in the blood and brain, and exacerbated cognitive impairment in APP/PS1 mice, suggesting that the liver-brain axis is implicated in AD neuropathology (Cheng et al., 2023).

However, there is contradicting evidence on whether NAFLD is associated with increased risk of AD in human subjects research. A recent study reported that NAFLD was associated with

an increased risk of dementia and AD in a population of adults over the age of 60 (Jeong et al., 2022). However, on the contrary, two recently published meta-analyses reported that NAFLD was not associated with AD or vascular dementia (Huang et al., 2023; Wang et al., 2022), but was associated with cognitive impairment (Wang et al., 2022). Despite these conflicting results, research in mice indicates that the relationship between diet, the liver and AD biomarkers, both in the periphery of the body and the central nervous system, should be further explored.

4g. The Effects of Diet on the Rodent Gut Microbiome

More recent research suggests that dysregulation of the gut microbiome contributes to chronic inflammation, cognitive impairment, and AD development (García-Montero et al., 2021; Pluta et al., 2020; Statovci et al., 2017). The human gastrointestinal tract is home to millions of microbes that play an essential role in both immune and cognitive function (Bonfili et al., 2022). For example, commensal bacteria, such as *Lactobacillus* and *Bifidobacterium*, produce neurotransmitters, such as acetylcholine, dopamine, and serotonin, that are necessary for normal cognitive processes (García-Montero et al., 2021; Statovci et al., 2017). Conversely, gram-negative bacteria, like *E. coli*, induce an inflammatory state that is harmful to both peripheral systems and the central nervous system (Statovci et al., 2017). The presence of gram-negative bacteria provokes excess secretion of pro-inflammatory cytokines and chemokines, and decreases protective short chain fatty acids (SCFAs) and neurotransmitters (Moreira et al., 2012; Statovci et al., 2017). For example, Moreira et al. (2012) demonstrated that a SFA rich diet increased the population of gram-negative bacteria and permeability in the intestines. Thus, the presence of harmful microbes disrupts microbiome homeostasis, engenders a leaky gut, and generates inflammation in the periphery of the body, and eventually the CNS (Statovci et al., 2017).

Diet plays a crucial role in microbiome composition, and the WD has been shown to provoke inflammation and microbiome dysbiosis by reducing essential phyla, such as *Bacteroidetes,* and increasing the production of phyla associated with disease, such as *Firmicutes* (Bruce-Keller et al., 2015; Jena et al., 2022; Li et al., 2018; Magnusson et al., 2015). For example, a fiber-deficient diet decreased essential *Bacteroidetes* and increased harmful *Firmicutes* in C57BL/6 mice (Yu et al., 2019). A balance of these two groups of phyla is imperative for proper gut microbiome function (Bruce-Keller et al., 2015). Indeed, Li and colleagues (2018) found that a WD increased peripheral inflammation and gram-negative bacteria, while reducing important commensal bacteria in C57BL/6 mice. The results of this study revealed that mice on a WD had significantly more *E. coli* and significantly less *Bifidobacterium* and *Lactobacillus* present in their gut microbiome, illustrating that the WD is harmful to the gut microbiome (Li et al., 2018).

Additionally, Jena et al. (2022) examined the effects of life long WD consumption on gut microbiome composition in C57BL/6 mice. Mice were administered a Western rodent diet from weaning to ten months of age, and at 8 months of age, they were provided probiotic supplementation (Jena et al., 2022). The results of this study revealed that the WD decreased commensal bacteria, and provoked the production of inflammation inducing bacteria, such as *Clostridiaceae* (Jena et al., 2022). However, *B. infantis* pro-biotic supplementation protected animals consuming the WD by promoting microbial diversity, decreasing *Firmicutes*, and reducing peripheral inflammation in the gut (Jena et al., 2022). Moreover, (Bruce-Keller et al., 2015) transplanted the microbiota from obese mice on either a high-fat diet or a low-fat diet, into healthy C57BL/6 mice, to examine the effects of microbiota transplants on cognition, behavior, and neuroinflammation. Interestingly, non-obese mice that received a microbiome transplant

from obese mice on a high-fat, Western rodent chow exhibited behavioral changes, cognitive deficits, and increased neuroinflammation, in comparison to mice that received microbiome transplants from animals on a low-fat diet (Bruce-Keller et al., 2015). Thus, this study suggests that the microbiome plays a key role in brain health, even in mice that have a healthy body weight (Bruce-Keller et al., 2015). Similarly, (Magnusson et al., 2015) also demonstrated that reduced microbial diversity and increased proliferation of harmful bacteria induced by the WD are related to cognitive impairment in C57BL/6 mice (Magnusson et al., 2015). Collectively, these studies demonstrate that the WD is harmful to the gut microbiome and the CNS.

As previously discussed, high fiber diets, like the MD, could potentially be used as an intervention strategy for microbiome dysregulation and cognitive dysfunction (Bourassa et al., 2016). Previous studies in C57BL/6 mice have demonstrated that plant-based diets, or fiber-supplemented diets, protect the gut microbiome in comparison to high-fat or low-fiber diets (Li et al., 2018; Liu et al., 2021; Yu et al., 2019). For example, Liu et al. (2021) examined the effects of a maternal high-fat diet, in comparison to a maternal high-fiber diet, in the gut microbiome of C57BL/6 mouse offspring. The results of this study revealed that the maternal high-fat diet decreased essential gut microbiota, such as *Bifidobacterium* and *Prevotella*, in comparison to a maternal high-fiber diet in offspring's gut microbiome, and the fiber deficient diet was also associated with cognitive dysfunction in C57BL/6 mice (Liu et al., 2021).

Additionally, Li and colleagues (2018) found that a PUFA rich, plant-based diet, increased microbial diversity and the production of essential bacteria, such as *Bifidobacterium* and *Lactobacillus*, in comparison to the WD (Li et al., 2018). Notably, the plant-based diet also improved gut microbiome health, anxiety-like behaviors, and spatial learning in C57BL/6 mice (Li et al., 2018). Therefore, the results of this study suggest that a plant-based diet not only

affects the gut microbiome, but also influences cognition and behavior, due to communication between the gut-brain axis (Li et al., 2018). Collectively, these studies demonstrated that plant-based foods and dietary fiber, which are both key elements of the MD, support a healthy gut microbiome. However, further research is needed to examine the relationship between microbial diversity and AD susceptibility following long-term consumption of a *comprehensive* MD, as this has not yet been thoroughly explored in wildtype mice.

CHAPTER 2: EXPERIMENT 1, THE LONG-TERM EFFECTS OF A COMPREHENSIVE, MEDITERRANEAN DIET VERSUS A TYPICAL AMERICAN DIET ON BEHAVIOR, SPATIAL MEMORY, PHYSIOLOGY, AND AD BIOMARKERS

1. Abbreviated Introduction

Although there are several variations of Western diets made for rodents, researchers have primarily utilized WDs providing approximately 20–50% kcal from carbohydrates, 15–20% kcal from protein, and 40–60% kcal from fat (Hintze et al., 2018; Więckowska-Gacek et al., 2021). However, these frequently used rodent diets are not truly representative of a typical American diet (TAD). For example, the majority of commercially available Western diets contain extremely exaggerated fat (Arnold et al., 2014; Denver et al., 2018; Guillemot-Legris, Masquelier, et al., 2016; Heyward et al., 2012; Julien et al., 2010; Lin et al., 2016; Pistell et al., 2010) or sugar content (Baranowski, Bott, & MacPherson, 2018; Gabriel et al., 2020). A recent review (Więckowska-Gacek et al., 2021) reported that the most common WDs administered to C57BL/6 mice provided approximately 42% (Liu et al., 2014; Rutkowsky et al., 2018), 45% (Denver et al., 2018), or 60% (Guillemot-Legris, Masquelier, et al., 2016; Heyward et al., 2012; Julien et al., 2010; Moser et al., 2018; Pistell et al., 2010) kcal from fat. However, the average American only consumes about 34–35% kcal from fat (Hintze et al., 2018; *Nutrient intakes from food and beverages: mean amounts consumed per individual, by gender and age, What We Eat in America, NHANES 2013–2014.* , 2016), and thus, more research is needed to examine the effects of the TAD on AD susceptibility in animals.

Also, prior studies have shown that supplementation of one or two Mediterranean dietary factors (e.g., olive oil) is neuroprotective in mice, yet there is limited research on the effects of a comprehensive Mediterranean rodent diet that mimics the typical human MD on AD biomarker susceptibility in wildtype mice. To address this gap in the scientific literature, the current study

examined the potential, protective effects of a *comprehensive* MD in comparison to a macronutrient-matched TAD. The two experimental diets were designed to focus on the following key factors: (1) mimic the typical macronutrient density ranges of carbohydrates, protein, and fat in human Mediterranean or American diets, (2) include diverse food sources that are typically consumed in either Mediterranean regions or the U.S., (3) match the macronutrient densities of both diets to control for energy availability.

The typical MD provides approximately 45–65 % kcal from carbohydrates, 15–20% kcal from protein, and 30–40% kcal from fat (Trichopoulou et al., 2003; Trichopoulou et al., 2014; Trichopoulou et al., 2005), while the average American consumes approximately 50% kcal from carbohydrates, 15.75% kcal from protein, and 34–35% kcal from fat (*Nutrient intakes from food and beverages: mean amounts consumed per individual, by gender and age, What We Eat in America, NHANES 2013–2014.* , 2016; Shan et al., 2019; Wright & Wang, 2010). Accordingly, both diets provided the same percentage of kilocalories from macronutrients, although they came from different food sources. For example, the MD included complex carbohydrates (e.g., brown rice flour) and unsaturated fatty acids (e.g., olive oil, fish oil, and flaxseed oil), while the TAD included refined carbohydrates (e.g., corn starch) and SFA (e.g., safflower oil, butter, and beef fat).

Hypotheses

Based on available evidence in rodent models, we hypothesized that early-life dietary pattern implementation and long-term consumption of a comprehensive MD would protect mice against physiological, biological, and cognitive markers of AD compared to the TAD, including: (1) excessive weight gain, (2) increased fat deposition in the abdomen and liver, (3) peripheral

inflammation, (4) $A\beta_{1-42}$ production in the cortex and hippocampus, and (5) behavioral and cognitive deficits.

2. General Materials and Methodology for Experiments 1–3

Subjects

The current experiments (1–3) utilized male and female wildtype, C57BL/6 mice, bred in the vivarium at Texas Christian University (TCU), from a breeding stock purchased from Jackson Laboratory (Bar Harbor, ME). All subjects were appropriately handled in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and the TCU Institutional Animal Care and Use Committee (IACUC Protocol #2021-14). Mice were housed in polycarbonate cages with pelleted, paper-chip bedding and compressed cotton square nestlets (LabSupply, Fort Worth, Texas) in groups of three to four, under a 12-hour light/dark schedule.

Experimental Diet Formulations

We designed two, macronutrient-matched rodent diets, the MD (#D21112902), and the TAD (#D21112903), that were created in pelleted form and purchased from Research Diets, Incorporated (New Brunswick, New Jersey). See Table 1 for experimental diet formulations and Table 2 for key ingredients. See Appendix A for complete experimental diet formulations and ingredient lists.

	MD Formula			TAD Formula		
	g%	**kcal%**	**gm**	**g%**	**kcal%**	**gm**
Protein	14.3	15.0	147.2	16.0	15.0	147.3
Carbs	48.0	50.0	495.3	53.7	50.0	495.5
Fat (Total)	14.8	35.0	153.2	16.6	35.0	153.1
SFA		6.7	29.5		17.0	74.4
MUFA		19.6	86.2		12.3	54.0
PUFA		5.6	24.4		3.2	14.1
Ratio *n*-6/*n*-3			2.0			15.6
Insoluble Fiber	8.3		85.4	5.4		50.0
Soluble Fiber	5.8		59.4	0.0		0.0

Table 1. Experimental Diet Formulas.

	MD Formula	TAD Formula
	Ingredients	**Ingredients**
Protein	Egg white, fish, soy protein	Casein
Carbs	Brown rice, wheat starch	Corn starch
Fat	Olive, fish, & flaxseed oil	Safflower oil, beef fat, butter
Insoluble Fiber	Cellulose	Cellulose
Soluble Fiber	Psyllium, inulin	

Table 2. Key Ingredients in the Experimental Diets.

Food and Body Weight Measures

We measured the amount of food consumed by each cage once per week. Every Friday morning, the total amount of food in the hopper of each cage was weighed and recorded. Subsequently, every Monday morning, the amount of food remaining in each hopper was weighed and recorded. Then, we measured the estimated, daily consumption for one individual mouse per cage with the following equation: the weight of food consumed (grams) divided by number of days of consumption (three days), divided by the number of animals per cage. Additionally, body weight was measured and recorded once a week.

Open Field Testing

At six months of age, mice underwent testing in open field to examine behavior and locomotor activity. Mice were tested in open field chambers (27 x 27 cm) that were enclosed

inside of ventilated, sound-attenuated boxes, with infrared beams to measure locomotor activity (Med Associates Incorporated, St. Albans, VT). Prior to testing, two zones were established, including the center zone (17.46 cm x 17.46 cm) and the remaining outer zone (423.39 cm^2). The time spent in the center zone versus the outer zone was utilized to measure anxiety-like behavior, as prior research has established that more time spent in the center zone is indicative of reduced anxiety-like behavior (Seibenhener & Wooten, 2015). During testing, mice were removed from their home cages, placed in the middle of the open field chamber, and given 10 minutes of uninterrupted movement. We utilized software (Med Associates Incorporated, St. Albans, VT) to measure the following dependent variables: vertical counts (rearing behavior), ambulatory distance traveled (centimeters), duration of time spent in the center zone (seconds), and the average speed of ambulatory episodes (centimeters per second).

Elevated Zero Maze

Mice were tested in the elevated zero maze to further measure anxiety-like behavior, as this paradigm has been verified by previous research (Shepherd et al., 1994). The elevated zero maze (platform 50 cm tall, circular diameter 60 cm) consisted of four alternating quadrants: two open quadrants and two quadrants enclosed by walls. At the beginning of the test, each mouse was placed in the open quadrant, facing a closed quadrant, and given five minutes of uninterrupted movement. A video camera was mounted on the ceiling to record the total time spent in the open quadrants versus the enclosed quadrants using EthoVision XT software (Noldus Information Technology, Leesburg, VA).

Object-Location Memory Task

Experimenters conducted the object-location memory task (OLM) to examine spatial learning and memory. Arena settings were created utilizing EthoVision XT software (Noldus Information Technology, Leesburg, VA) and recorded with a mounted video camera in the

ceiling. OLM consisted of habituation (2 sessions, 4 hours apart, no objects), training (2 sessions, 4 hours apart, two identical objects), and testing, in which one of the identical objects was moved to the opposite corner (novel spatial location). The objects were created according to guidelines from prior research, and consisted of 100mL syringes filled with cement, with copper coiled around it (4cm width X 4.5cm length X 17.5cm height). Objects were counterbalanced in all four chambers, and lights above the testing boxes were kept between 45–48 lux. During each session, mice were placed in center of the chamber in the same orientation and allowed ten minutes to freely explore. See Figure 5 for timeline.

Experimenters recorded the time (s) spent exploring each object, and object exploration was recorded if the mouse was facing the object in close proximity, with its nose in the predetermined zone, within three centimeters surrounding the object. Following testing, the percentage of time spent exploring the object in the novel location was calculated utilizing the following equation: novel object location time/[novel object location time + old object location time] x 100, and this formula was adopted from (Heyward et al., 2012). Experimenters also measured the percentage time exploring both objects during training, to ensure that there was no object location preference.

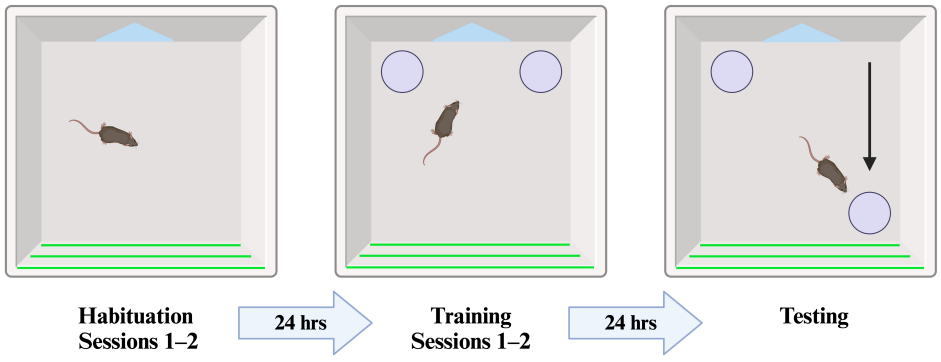


Figure 5. Object-Location Memory Task. During each session, mice were placed in the OLM

chamber and provided ten minutes of uninterrupted movement. During the training session, experimenters recorded the percentage of time that mice spent exploring two, identical objects. During testing, one object was moved to the opposite corner of the chamber, and the percentage of time exploring the object in the novel location was measured.

3. Materials and Methodology for Experiment 1

Experimental Design and Timeline

At post-natal day 21, mice were weaned and randomly assigned to one of two experimental diets: a Mediterranean diet (MD) or typical American diet (TAD) (*n*'s = 21) for six months, with access to food and water *ad libitum*. At six months of age, mice underwent behavioral testing. At six months and 21 days of age, tissue and serum were collected. See Figure 6 for experimental timeline.

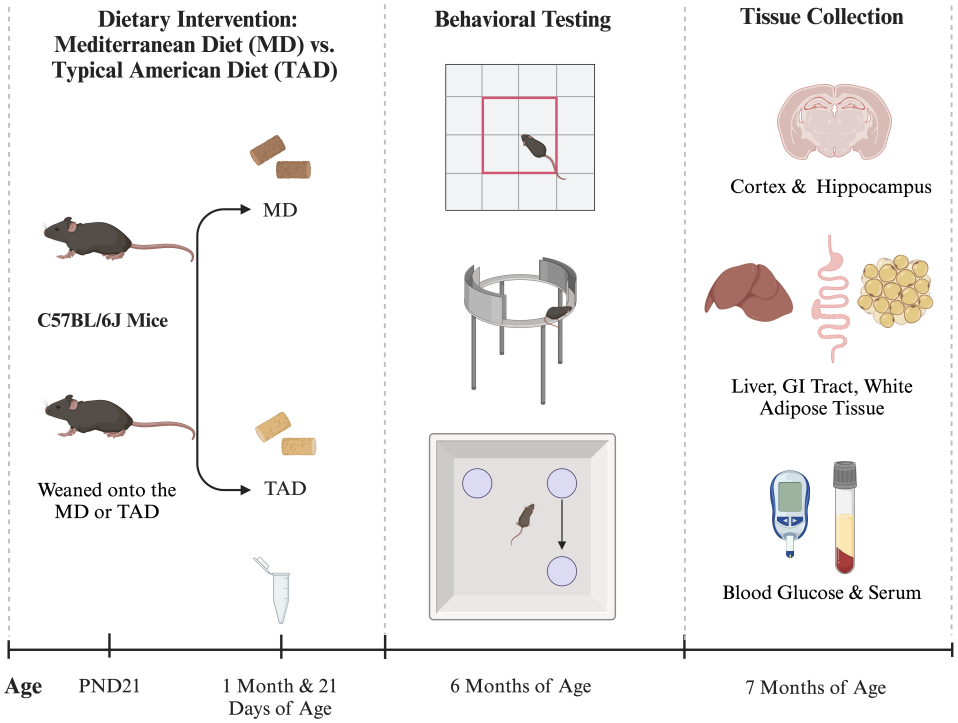


Figure 6. Experimental Timeline for Experiment 1.

Fecal Sample Collections

Fecal samples were collected at three different timepoints: at weaning (PND21), one month of diet and six months of diet, and stored at -80 degrees Celsius. Microbiome analyses were conducted by our collaborators in the Allen Lab (University of North Texas, Health Science Center, Fort Worth, Texas).

Tissue and Serum Collection

After six months of the prescribed diets, mice underwent fasting for 6 hours, prior to euthanasia. Trunk blood was collected, and whole blood was utilized to measure fasting blood glucose (Cholestech, Alere Inc., Hayward, Ca). The remaining blood samples were placed on ice for 15 minutes and then kept at room temperature for 30 minutes. Subsequently, blood was centrifuged (2,000 x g) for 10 minutes, and serum was collected and stored at -80ºC. Following blood collection, experimenters removed the liver, spleen, and white adipose tissue, recorded weights of the tissue samples (grams), and stored them in neutral buffered formalin at 4ºC. Additionally, both hemispheres of the frontal cortex and hippocampus were collected and lysed in 250 µL of Proprep (PRO-PREP, Bulldog Bio, Portsmouth, NH), supplemented with additional protease and phosphatase inhibitors (Sigma Aldrich, Burlington, Massachusetts). All tissue lysates were rapidly frozen on dry-ice following the collection process and stored at -80ºC.

Peripheral Cytokine Analysis

Concentrations of inflammatory Mediators were quantified in serum to examine the effects of diet on peripheral inflammation utilizing a VPLEX Custom Mouse Cytokine Pro-Inflammatory Panel 1 multiplexing kit (Meso Scale Diagnostics, Rockville, MD). The custom kit was created to measure the following inflammatory mediators in serum: mouse TNF-α, IL-1β, IL-6, IL-10, and IFN- γ. First, we diluted serum samples in plate at a 1:1 dilution with a proprietary diluent. Samples were then incubated at room temperature for two hours with horizontal shaking

at 750 rpm. Following sample incubation, all wells were washed three times, and a detection antibody solution was added to each well. Following a two-hour incubation period, all wells were washed three times, and read buffer was subsequently added to each well. Electrochemiluminescent signal was read using a QuickPlex SQ 120 instrument (Meso Scale Diagnostics, Rockville, MD). Sample replicates with an intra-assay coefficient variation of over 25% were excluded from the analysis, as advised by MSD technical support.

Liver Histology

a. Paraffin Sectioning

Paraffin embedded samples were sectioned at room temperature using a microtome (Leica RM2235, Wetzlar, Germany) at eight and ten micrometers. Sections were then placed in a 42°C water bath (Leica HI1210, Wetzlar, Germany), fixed onto StatLab Millenia Command Adhesion Slides/W/90 (StatLab, McKinney, Texas), and set to dry overnight.

b. H&E Staining

Hematoxylin and eosin (H&E) staining was performed to examine the histological morphology of the liver, as this is a commonly used method for *in vivo* experiments focusing on hepatic steatosis (Cui et al., 2017). Following paraffin processing and sectioning, prepped slides were washed in Sub-X Clearing Medium (Leica, Wetzlar, Germany) for four minutes (three repetitions), and then rehydrated in 100% ethanol for two minutes (three repetitions), 100% ethanol for one minute, and then 95% ethanol for one minute.

Subsequently, the slides were washed in water for a minute and then stained in hematoxylin (Leica Surgipath SelecTech Hematoxylin 560, Wetzlar, Germany) for five minutes. Following staining, slides were continuously washed indirectly for three minutes before being placed in differentiate (Leica Surgipath SelecTech Define, Wetzlar, Germany). After, slides were rinsed in water for one minute, and then placed in blue buffer (Leica Surgipath SelecTech Blue

Buffer 8, Wetzlar, Germany) for 30 minutes. The cytoplasm was stained red with Eosin (Lecia Surgipath SelecTech Alcohol Eosin Y 515, Wetzlar, Germany), and subsequently rinsed in water for one minute.

Next, the slides were dipped in water before starting the dehydration process in 95% ethanol for two minutes, and then 100% ethanol for one minute in three separate washes, followed by the same process with Sub-X, but for two minutes each. The slides were then layered with SubMount Mounting Medium (StatLab, McKinney, Texas), and a coverslip. Images were taken with a Nikon DS-Fi1 camera (Nikon Instruments, Toyko, Japan), and Nikon Eclipse 90i microscope (Nikon Instruments, Toyko, Japan), at a magnification of 40x. White balance was performed using the software NIS-Elements AR 4.60.00 (Nikon Instruments, Toyko, Japan) by selecting unstained area as the white point. Images were converted from RGB image to 8-bit image using Image J (version 1.53k). Autothresholding was performed and the Triangle Method of segmentation was selected as the closest match to stained vs unstained areas. In this method, areas stained with hematoxylin or eosin are shown as black and unstained area is shown as white. Images were then measured for percentage of image that is unstained. This value represents vascular and ductal lumen and areas where tissues may have been damaged in the processing and sectioning. More importantly, this value also represents areas inside the cells that once contained lipids that were removed in the paraffin processing of the tissue.

c. Cryosection

In addition to H & E staining, experimenters conducted Oil Red O Staining to examine lipids in the liver tissue. Livers were kept refrigerated at 4 ºC in vials of 30% sucrose in phosphate buffered saline (PBS) until they sank to the bottom of the conical tube (about four days). Second, tissues were placed in a mold filled with optimal cutting temperature compound (OCT), and stored at -20°C. Once frozen, the tissue block was removed from the mold and

mounted onto the cutting disk of the cryostat (Leica CM1900, Wetzlar, Germany) for sectioning at -20°C. Sections were collected at 8µm and 10µm thickness. Sections were transferred to a room temperature microscope slide and stored at room temperature.

d. Oil Red O Staining

Staining was conducted utilizing an Oil Red O Stain Kit (StatLab, McKinney, Texas). Slides were immersed in propylene glycol for two minutes, followed by Oil Red O at 60°C for six minutes. Subsequently, the slides were immersed in 85% propylene glycol for one minute. The slides were rinsed with running DI water before immersion in Modified Mayer's Hematoxylin (StatLab, McKinney, Texas) for one minute. Next, the slides were rinsed with water for one minute, and mounted with coverslips using aqueous mounting medium (StatLab, McKinney, Texas). Images were taken with a Nikon DS-Fi1 camera (Nikon Instruments, Toyko, Japan), and Nikon Eclipse 90i microscope (Nikon Instruments, Toyko, Japan), at a magnification of 40x, using the software NIS-Elements AR 4.60.00 (Nikon Instruments, Toyko, Japan).

$A\beta_{1-42}$ ELISA

All tissue samples were centrifuged at 15,000 rpm for 40 minutes and clear lysates were collected. Protein assays (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA) were conducted to calculate the protein concentrations in all brain lysates. A mouse $A\beta_{1-42}$ ELISA (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts) was conducted to measure soluble $A\beta_{1-42}$ from both the cortex and hippocampus. The protein concentrations of the brain lysates were standardized, and then diluted in incubation buffer (1:2 dilution). Following dilutions, 100 µL of sample lysates and standards were plated in duplicates in a 96-well, pre-coated plate with capture antibody. Following two hours of incubation, the plates were decanted and washed four times with wash buffer (1X). Then, each well was treated with $A\beta_{1-42}$ detection antibody solution and incubated for one hour. Subsequently, the plates were washed again and

treated with 100 µL of HRP-tagged detection antibody (Anti-Rabbit IgG). Following 30 minutes of incubation, the plates were washed and treated with 100 µL stabilized chromogen (tetramethylbenzidine) and incubated in the dark for an additional 30 minutes. Finally, 100 mL of stop solution was added to all wells, and the plate was read at an absorbance of 450nm (BMG LabTech FLUOstar Omega, Cary, North Carolina). Sample replicates with an intra-assay coefficient variation over 25% were excluded from the analysis.

Statistical Analyses

All data were analyzed with Statistical Package for Social Sciences (SPSS; Version 29.0, IBM, Armonk, NY). Normality was assessed prior to running statistical analyses, and transformations were performed to correct for any violations of homogeneity of variance. Independent samples t-tests, two-tailed, and mixed-design analysis of variance (ANOVAs) were conducted to examine behavioral and biological dependent measures. We further examined the relationships between several dependent variables utilizing the Pearson product-moment correlation coefficient. All outliers were detected utilizing SPSS's interquartile range, and for all analyses, an alpha level of $p \leq 0.05$ was considered significant.

4. Results for Experiment 1

Average Food and Kilocalorie Consumption in Male Mice

Two mixed-design ANOVAs revealed a significant main effect of diet duration on daily food consumption (grams), $F(5,60) = 9.988$, $p \leq 0.001$, $\eta_p^2 = 0.454$, and kilocalorie intake $F(5,60) = 9.988$, $p \leq 0.001$, $\eta_p^2 = 0.454$, in male mice following six months of diet. Additionally, there was a significant main effect of diet condition (MD vs. TAD) on food consumption, $F(1,12) = 6.276$, $p = 0.028$, $\eta_p^2 = 0.343$, and kilocalorie intake $F(1,12) = 6.276$, $p = 0.028$, $\eta_p^2 = 0.343$, in which male mice assigned to the MD consumed more food and kilocalories than those assigned to the TAD.

These results were further supported by a significant diet x diet duration interaction, $F(5,60) = 2.673$, $p = 0.030$, $\eta_p^2 = 0.182$, such that diet consumption and kilocalorie intake significantly increased as the mice aged and reached adulthood. Further analyses utilizing Tukey's LSD revealed a significant difference in daily food consumption between diet conditions following 3 months ($p = 0.028$) and 6 months ($p = 0.025$) of diet administration, in which mice on the MD consumed significantly more food and kilocalories than those on the TAD. See Figure 7.

Average Food and Kilocalorie Consumption in Female Mice

A mixed-design ANOVA revealed a significant main effect of diet duration on daily food consumption (grams) over the course of six months of diet administration in female mice, $F(5,30) = 13.689$, $p \leq 0.01$, $\eta_p^2 = 0.695$. Further, there was no significant main effect of diet condition (MD vs. TAD) on food consumption, $F(1,6) = 3.648$, $p = 0.105$, $\eta_p^2 = 0.378$. Additionally, a mixed-design ANOVA revealed a significant main effect of diet duration on daily kilocalorie intake over the course of six months, $F(5,30) = 13.689$, $p \leq 0.01$, $\eta_p^2 = 0.695$, but not a significant main effect of diet condition (MD vs. TAD) on kilocalorie intake, $F(1,6) = 3.648$, $p = 0.105$, $\eta_p^2 = 0.378$. At four and six months of diet consumption, the data violated homogeneity of variance for both daily food consumption and kilocalorie intake, necessitating log transformation. Following transformations, the data still violated homogeneity of variance, and a more conservative p-value was adopted. See Figure 8.

Body Weight in Male Mice

A mixed-design ANOVA revealed a significant main effect of diet duration (months) on body weight (grams) over the course of six months of diet administration in male mice, $F(6,240) = 2013.004$, $p \leq 0.001$, $\eta_p^2 = 0.981$. Additionally, there was a significant main effect of diet condition (MD vs. TAD) on male body weight, $F(1,40) = 16.365$, $p = \leq 0.001$, $\eta_p^2 = 0.290$, such that mice on

the MD weighed less than those on the TAD. These results were further supported by a significant interaction, $F(6,240) = 21.843$, $p \leq 0.001$, $\eta_p^2 = 0.353$. Further analyses utilizing Tukey's LSD revealed that there was a significant difference in body weight between diet conditions following 3,4, 5, and 6 months of diet (ps < 0.016), such that males on the MD weighed less than those on the TAD. See Figure 7.

Body Weight in Female Mice

A mixed-design ANOVA revealed a significant main effect of diet duration (months) on body weight (grams) over the course of six months of diet administration in female mice, $F(6,198) = 913.282$, $p \leq 0.001$, $\eta_p^2 = 0.965$. However, there was no significant main effect of diet condition (MD vs. TAD) on female body weight, $F(1,33) = 0.037$, $p = 0.849$, $\eta_p^2 = 0.001$. Months 4, 5, and 6 of diet administration violated homogeneity of variance, and thus a log transformation was performed. However, the transformation did not correct this error, and a more conservative p-value was adopted. Additionally, an outlier was outside of the interquartile range, determined by SPSS, and removed from the analysis. See Figure 8.

Organ and White Adipose Tissue Weight in Male Mice

An independent samples t-test revealed a significant difference in white adipose tissue weight, $t(27) = -9.194$, $p \leq 0.001$, and liver weight, $t(27) = - 4.355$, $p \leq 0.001$, in which mice on the TAD had heavier white adipose tissue and livers than those on the MD. The magnitude of the difference in the means of white adipose tissue weights (mean difference = -1.397, 95% CI [-1.085, - 1.709]) was large (eta squared = 0.758), and the magnitude of the difference in the means of liver weights (mean difference = -0.523, 95% CI [-0.277, - 0.769]) was large (eta squared = 0.413). However, there was no difference in spleen weight, $t(33) = -1.583$, $p = 0.123$. See Figure 9.

Organ and White Adipose Tissue Weight in Female Mice

An independent samples t-test did not reveal a significant difference in spleen weight, $t(27) = 0.787$, $p = 0.438$, liver weight, $t(27) = 0.376$, $p = 0.710$, nor white adipose tissue weight, $t(27) = 0.042$, $p = 0.967$, in female mice. See Figure 10.

Fasting Blood Glucose in Males

An independent samples t-test revealed that mice on the TAD had significantly higher blood glucose (mg/dL) levels in comparison to those on the MD, $t(10) = -2.429$, $p = 0.036$, two-tailed. The magnitude of the difference in the means (mean difference = -121.333, 95% CI [-10.026, -232.640]) was large (eta squared = 0.371). See Figure 9.

Fasting Blood Glucose in Females

An independent samples t-test failed to reveal a difference in fasting blood glucose (mg/dL) levels between diet conditions in female mice, $t(13) = -1.665$ $p = 0.120$, two-tailed. It is important to note that we were not able to collect data from each female mouse due to malfunction of the Cholestech machine. See Figure 10.

Liver Histology in Males

We imaged four, randomly selected liver sections from three male mice in each diet condition (MD or TAD). An independent samples t-test revealed a significant difference in the percentage of pixelated area that was unstained following H & E staining, such that male mice on the MD had less percentage of pixelated area unstained compared to those on the TAD, $t(21) = 39.173$, $p \leq 0.001$. The data violated homogeneity of variance, and a reflect and square root transformation was performed to correct this. The magnitude of the difference in the means of unstained area in the liver

(mean difference = -22.486, 95% CI [-21.368, - 21.347]) was large (eta squared = 0.986). See Figure 11.

Liver Histology in Females

We imaged four, randomly selected liver sections from three female mice in each diet condition (MD or TAD). An independent samples t-test revealed a significant difference in the percentage of pixelated area that was unstained following H & E staining, such that female mice on the MD had less percentage of pixelated area unstained compared to those on the TAD, $t(22) = -46.58$, $p \leq 0.001$. The magnitude of the difference in the means of area unstained (mean difference = -26.884, 95% CI [-25.687, - 28.081]) was very large (eta squared = 1.010). See Figure 12.

Peripheral Cytokine Production in Serum of Male Mice

An independent samples t-test revealed that mice on the TAD produced significantly more TNF-α (pg/mL) compared to those on the MD, $t(32) = -3.790$, $p \leq 0.001$. One sample was removed from the analysis due to an intra-assay coefficient of variation (CV) value over 25%, recommended by MSD's technical team. The magnitude of the difference in the means (mean difference = -2.12, 95% CI [-3.253, -0.979]) was large (eta squared = 0.31). Additionally, the difference in IL-1β (pg/mL) production between diet conditions approached significance, $t(23) = -1.809$, $p = 0.084$. Six samples were removed from the analysis due to CV values over 25%. Additionally, three outliers were removed from the analysis because they fell outside of the interquartile range determined by SPSS. The magnitude of the difference in the means (mean difference = -0.07967, 95% CI [0.0115, -0.171) was moderate (eta squared = 0.125). Further, there was no significant difference in IL-10 (pg/mL) production between diet conditions, $t(29) = -0.818$, $p = 0.420$. Three outliers were removed from the analysis because they fell outside of the interquartile range determined by SPSS. Finally, an independent samples t-test failed to reveal a significant difference in IFN- γ (pg/mL) production

between diet conditions, $t(30) = 0.040$, $p = 0.968$. Two samples were removed from the analysis due to CV values over 25%. See Figure 13.

Peripheral Cytokine Production in Serum of Female Mice

An independent samples t-test was conducted to compare the quantity of TNF-alpha (pg/mL) in female mice serum following 6 months of MD or TAD diet consumption. Female mice on the TAD produced significantly more TNF-alpha (pg/mL) in comparison to those on the MD, $t(19) = -3.903$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was conducted. Following transformation, the data still violated homogeneity of variance, and thus a more conservative p-value was adopted, $p \leq 0.01$. The magnitude of the difference in the means (mean difference = -2.604, 95% CI [-1.043, - 4.164]) was large (eta squared = 0.445). One sample with a CV over 25% was removed from the analysis. Four outliers were detected outside of SPSS's interquartile range and were removed from the analysis. Additionally, an independent-samples t-test revealed that females on the MD produced significantly more IL-1β (pg/mL) compared to those on the TAD, $t(21) = 3.198$, $p = 0.004$. The magnitude of the difference in the means (mean difference = 0.098, 95% CI [0.162, 0.035]) was large (eta squared = 0.328). One outlier was removed from the analysis. Further, there was no significant difference in IL-10 (pg/mL), $t(24) = 0.112$, $p = 0.912$, IL-6 (pg/mL), $t(24) = -0.949$, $p = 0.352$, or IFN- γ (pg/mL), $t(21) = 0.360$, $p = 0.722$, between females on the MD and the TAD. Three outliers were removed from the analysis for IFN- γ. See Figure 14.

$A\beta_{1–42}$ Production in the Cortex and Hippocampus of Male Mice

An independent samples t-test revealed a significant difference in $A\beta_{1–42}$ (pg/mg) in the cortex, $t(22) = -3.067$, $p = 0.006$, in which male mice on the MD had less $A\beta_{1–42}$ in the cortex, in comparison to those on the TAD. The data violated homogeneity of variance, and a log transformation was performed to correct this. The magnitude of the difference in the means (mean

difference = -9.63, 95% CI [-3.10, - 16.16,]) was very large (eta squared = 0.299). Approximately 30% of variance was explained by diet. Two outliers were outside of the interquartile range determined by SPSS and removed from the analysis.

An independent samples t-test revealed a significant difference in $A\beta_{1–42}$ (pg/mg) in the hippocampus, such that mice on the MD had less hippocampal $A\beta_{1–42}$ compared to those on the TAD, $t(29) = -2.103$, $p = 0.044$. The magnitude of the difference in the means (mean difference = -4.522, 95% CI [- 0.124, -8.920]) was moderate (eta squared = 0.132). Three outliers were removed from the analysis. Additionally, two samples were removed from the analysis due to intra-assay CV values over 25%. See Figure 15.

$A\beta_{1–42}$ Production in the Cortex and Hippocampus of Female Mice

An independent samples t-test was conducted to compare the amount of soluble $A\beta_{1–42}$ in the cortex of female C57BL/6 mice following six months of the MD or TAD. There was a significant difference in $A\beta_{1–42}$ (pg/mg of protein) in female mice, $t(21) = -2.922$, $p = 0.008$, such that females on the MD had less $A\beta_{1–42}$ in the cortex, in comparison to females on the TAD. These data violated homogeneity of variance, and thus a log transformation was conducted to correct this. After running a transformation, the data still violated homogeneity of variance, and we adopted a more conservative p-value, $p \leq 0.01$. The magnitude of the difference in the means (mean difference = -10.162, 95% CI [- 18.503, -1.822]) was large (eta squared = 0.234). Approximately 23% of variance was explained by diet condition. One outlier was detected and removed from the analysis. Additionally, one sample was removed from the dataset because it had a CV value over 25%.

In contrast to the cortex, an independent-samples t-test failed to reveal a significant difference in $A\beta_{1–42}$ (pg/mg of protein) in female mice, $t(24) = -1.308$, $p = 0.203$. The magnitude of the difference in the means (mean difference = -2.83709, 95% CI [-7.313, 1.63834]) was moderate (eta

squared = 0.067). One outlier was detected and removed from the analysis. Additionally, two samples were removed from the analysis due to CV values over 25%. See Figure 16.

Open Field Behavior in Male Mice

a. Vertical Counts in Male Mice

An independent samples t-test failed to reveal a significant difference in vertical counts during the first five minutes (bin 1) of testing, $t(27) = 0.163$, $p = 0.872$. However, an independent samples t-test revealed a significant difference in vertical counts during the last five minutes (bin 2) of testing, $t(27) = 3.558$, $p \leq 0.001$, and during the total testing time $t(27) = 2.767$, $p \leq 0.01$, such that mice on the MD exhibited significantly more vertical counts than mice on the TAD. The magnitude of the difference in the means during the total testing time (mean difference = 14.111, 95% CI [24.573, 3.648]) was very large (eta squared = 0.221). Three outliers were detected utilizing SPSS and were removed from the analysis.

Additionally, a mixed-design ANOVA failed to reveal a significant main effect of time duration (first five minutes vs. last five minutes of testing) on the number of vertical counts in male mice, $F(1,27) = 0.182$, $p = 0.673$, $\eta_p^2 = 0.007$. However, there was a significant main effect of diet condition (MD vs. TAD) on vertical counts, $F(1,27) = 7.658$, $p = \leq 0.01$, $\eta_p^2 = 0.221$, such that mice on the MD exhibited significantly more vertical counts compared to mice on the TAD. Further analyses utilizing Tukey's LSD revealed that there was a significant difference between groups during the last five minutes of testing, in which mice on the MD exhibited more vertical counts than those on the TAD. Three outliers were excluded from the analysis. See Figure 6.

b. Distance Traveled in Male Mice

An independent samples t-test failed to reveal a significant difference in ambulatory distance traveled during the first five minutes (bin 1) of testing, $t(30) = 1.153$, $p = 0.258$. However, there was

a significant difference in distance traveled during the last five minutes (bin 2) of testing, $t(30) = 2.136$, $p = 0.041$. The magnitude of the difference in the means during the last five minutes of testing time (mean difference = 131.194, 95% CI [256.609, 5.77998]) was large (eta squared = 0.132). Additionally, the difference in total ambulatory distance traveled approached significance for the total testing time $t(30) = 1.867$, $p = 0.072$. The magnitude of the difference in the means during the total testing time (mean difference = 213.878, 95% CI [447.887, - 20.130]) was moderate (eta squared = 0.104). Further, when analyzing the total ambulatory distance traveled in the center zone, the difference in the distance traveled approached significance, $t(30) = 1.961$, $p = 0.059$. There was no difference in the total ambulatory distance traveled in the outer zone, $t(30) = 0.702$, $p = 0.488$. Additionally, a paired-samples t-test revealed that mice on the MD traveled a significantly farther distance in the center zone compared to the outer zone, $t(16) = -2.777$, $p = 0.013$, whereas there were no differences in the amount of distance traveled between the center and outer zone, $t(14) = -0.954$, $p = 0.356$.

c. Time Spent in the Center Zone of Open Field in Male Mice

An independent samples t-test failed to reveal a significant difference in time spent in the center zone during the first five minutes (bin 1) of testing, $t(27) = 1.764$, $p = 0.089$, during the last five minutes of testing (bin 2), $t(27) = 1.200$, $p = 0.241$, or in the total duration of testing, $t(27) = 1.748$, $p = 0.092$.

d. Average Speed in Male Mice

An independent samples t-test failed to reveal a significant difference in the average speed (cm/s) during the first five minutes (bin 1) of testing, $t(28) = 0.779$, $p = 0.443$, during the last five minutes of testing (bin 2), $t(28) = 1.430$, $p = 0.164$, or during the total testing time, $t(28) = 1.347$, $p = 0.189$. See Figure 17.

Open Field Behavior in Female Mice

a. Vertical Counts in Female Mice

An independent samples t-test failed to reveal a significant difference in vertical counts during the first five minutes (bin 1) of testing, $t(24) = -0.951$, $p = 0.351$, the last five minutes of testing (bin 2), $t(24) = 0.456$, $p = 0.652$, or the total testing time (10 minutes total), $t(24) = -0.255$, $p = 0.801$, between female mice on the MD and the TAD.

b. Distance Traveled in Female Mice

An independent samples t-test failed to reveal a significant difference in ambulatory distance traveled during the first five minutes (bin 1) of testing, $t(23) = -0.563$, $p = 0.579$, the last five minutes (bin 2) of testing, $t(23) = -0.168$, $p = 0.868$, or during the total time testing (10 minutes), $t(23) = -0.406$, $p = 0.688$, between female mice on the MD and the TAD. One outlier was excluded from the analyses because it fell outside of the interquartile range determined by SPSS.

c. Time Spent in the Center Zone of Open Field in Female Mice

An independent samples t-test revealed a significant difference in the time spent in the center zone during the first five minutes (bin 1) of testing, $t(21) = 2.208$, $p = 0.038$, in which females on the MD spent more time in the center of the open field compared to those on the TAD. However, there was no significant difference in the time spent in the center zone during the last five minutes of testing (bin 2), $t(21) = 0.085$, $p = 0.933$, or the total duration of testing, $t(21) = 1.454$, $p = 0.161$. Data for bin 2 and total testing time data violated homogeneity of variance, and a log transformation was performed to correct this. Following the transformation, the data still violated homogeneity of variance and a more conservative p-value was adopted. Three outliers were removed from the analyses.

d. Average Speed in Female Mice

An independent samples t-test failed to reveal a significant difference in the average speed (cm/s) during the first five minutes (bin 1) of testing, $t(25) = 1.640$, $p = 0.113$, between female mice on the MD and the TAD. However, there was a significant difference in the average speed during the last five minutes of testing (bin 2), $t(25) = 2.516$, $p = 0.019$, and during the total testing time (10 minutes), $t(25) = 2.276$, $p = 0.032$, in which females on the MD traveled significantly faster than those on the TAD. The magnitude of the difference in the means during the last five minutes of testing (bin 2) (mean difference = 9.591, 95% CI [17.441, 1.740]) was large (eta squared = 0.202). The magnitude of the difference in the means during the total testing time (mean difference = 7.516, 95% CI [14.316, 0.716]) was large (eta squared = 0.172). See Figure 18.

Elevated Zero Maze Behavior in Male Mice

An independent samples t-test revealed that male mice on the MD spent significantly more time in the open quadrants of the elevated zero maze in comparison to those on the TAD, $t(31) = 3.121$, $p = 0.004$. The data violated homogeneity of variance, and a log transformation was conducted to correct this. The magnitude of the difference in the means (mean difference = 0.214, 95% CI [0.354, 0.743]) was large (eta squared = 0.239). One outlier was detected by SPSS and excluded from the analysis. See Figure 17.

Elevated Zero Maze Behavior in Female Mice

An independent samples t-test failed to reveal a significant difference in the time spent in the open quadrants of the elevated zero maze between female mice on the MD and the TAD, $t(26) = -0.573$, $p = 0.571$. See Figure 18.

Spatial Learning and Memory in OLM in Male Mice

An independent samples t-test failed to reveal a significant difference in the percentage of time spent exploring the new object location during training, $t(18) = -0.787$, $p = 0.441$. The magnitude of the difference in the means (mean difference = -5.607, 95% CI [9.355, -20.569]) was small (eta squared = 0.033). However, an independent samples t-test revealed that mice on the MD spent significantly more time exploring the object moved to the new location, compared to mice on the TAD, during testing, $t(18) = 2.259$, $p = 0.037$. The magnitude of the difference in the means (mean difference = 21.919, 95% CI [42.305, 1.532]) was large (eta squared = 0.221). See Figure 19.

Spatial Learning and Memory in OLM in Female Mice

An independent samples t-test failed to reveal a significant difference in the percentage of time spent exploring the new object location during training, $t(17) = 0.557$, $p = 0.585$, between diet conditions in female mice. The magnitude of the difference in the means (mean difference = 1.232, 95% CI [-3.437, 5.901]) was small (eta squared = 0.018). Additionally, there was no significant difference in the percentage of object exploration time in the novel location during testing in female mice, $t(17) = 0.638$, $p = 0.532$. The magnitude of the difference in the means (mean difference = 5.409, 95% CI [-12.486, 23.305]) was small (eta squared = 0.023). See Figure 20.

Correlation Analyses in Male Mice

a. Examining the Relationship Between Spatial Memory and $A\beta_{1–42}$ in Male Mice

The relationship between cortical $A\beta_{1–42}$ (pg/mg of protein) and the percentage of time exploring the object moved to the new location during OLM testing was investigated using a Pearson product-moment correlation coefficient. There was a medium, negative correlation between the two variables, $r = -.498$, $n = 15$, $p = 0.059$. The correlation approached significance, suggesting that high levels of $A\beta_{1–42}$ were associated with decreased object exploration in the

new location during testing. See Figure 19. We also investigated the relationship between hippocampal $A\beta_{1–42}$ (pg/mg of protein) and the percentage of time exploring the object in the new location, and found a medium, negative correlation, however it was not significant, $r = -.429$, $n = 15$, $p = 0.111$.

b. Examining the Relationship Between $A\beta_{1-42}$, White Adipose Fat & Liver Weight, and TNF- α in Male Mice

We further examined the relationship between $A\beta_{1–42}$ and variables associated with modifiable risk factors for AD. There was a large correlation between white adipose fat deposition in the abdomen and cortical $A\beta_{1–42}$, $r = 0.516$, $n = 16$, $p = 0.041$, indicating that increased white adipose fat is associated with higher $A\beta_{1–42}$ production. We also examined the relationship between hippocampal $A\beta_{1–42}$ (pg/mg of protein) and white adipose fat, and only discovered a medium correlation that was not significant, $r = 0.348$, $n = 18$, $p = 0.157$.

Additionally, we investigated the relationship between white adipose fat, liver weight, and TNF- α production. There was a large correlation between white adipose fat and serum TNF- α, $r = 0.626$, $n = 22$, $p = 0.002$, as well as liver weight and serum TNF-α, $r = 0.516$, $n = 22$, $p = 0.014$. Furthermore, as excess body weight is known as a key modifiable risk factor for AD (Lancet Commission), we examined the relationship between body weight and serum TNF- α. There was a large correlation between body weight following six months of diet administration, and serum TNF- α, $r = 0.645$, $n = 34$, $p \leq 0.001$, indicating that excess body weight is associated with increased peripheral inflammation.

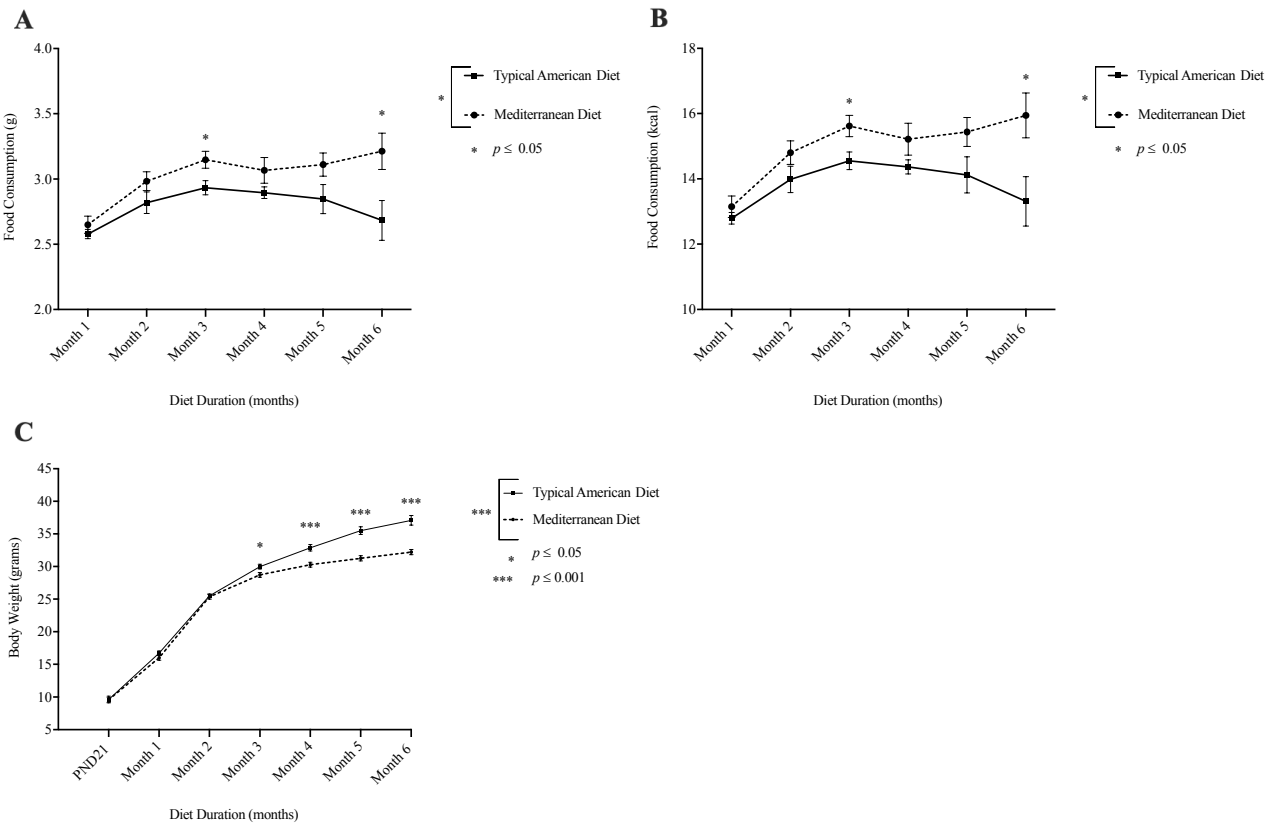


Figure 7. Food Consumption and Body Weight Throughout Diet Administration in Males. (A) A mixed-design ANOVA revealed a significant main effect of diet condition (MD vs. TAD) on food consumption, such that mice on the MD consumed more food than those on the TAD, $p = 0.028$, N's = 7. (B) Mice assigned to the MD consumed more kilocalories per day than those on the TAD, $p = 0.028$, N's = 21. (C) There was a significant main effect of diet condition on body weight, $p \leq 0.001$, in which males on the MD weighed less than those on the TAD, N's = 21. Bars represent ± SEM. Significant differences ($p < 0.05$) are designated by *.

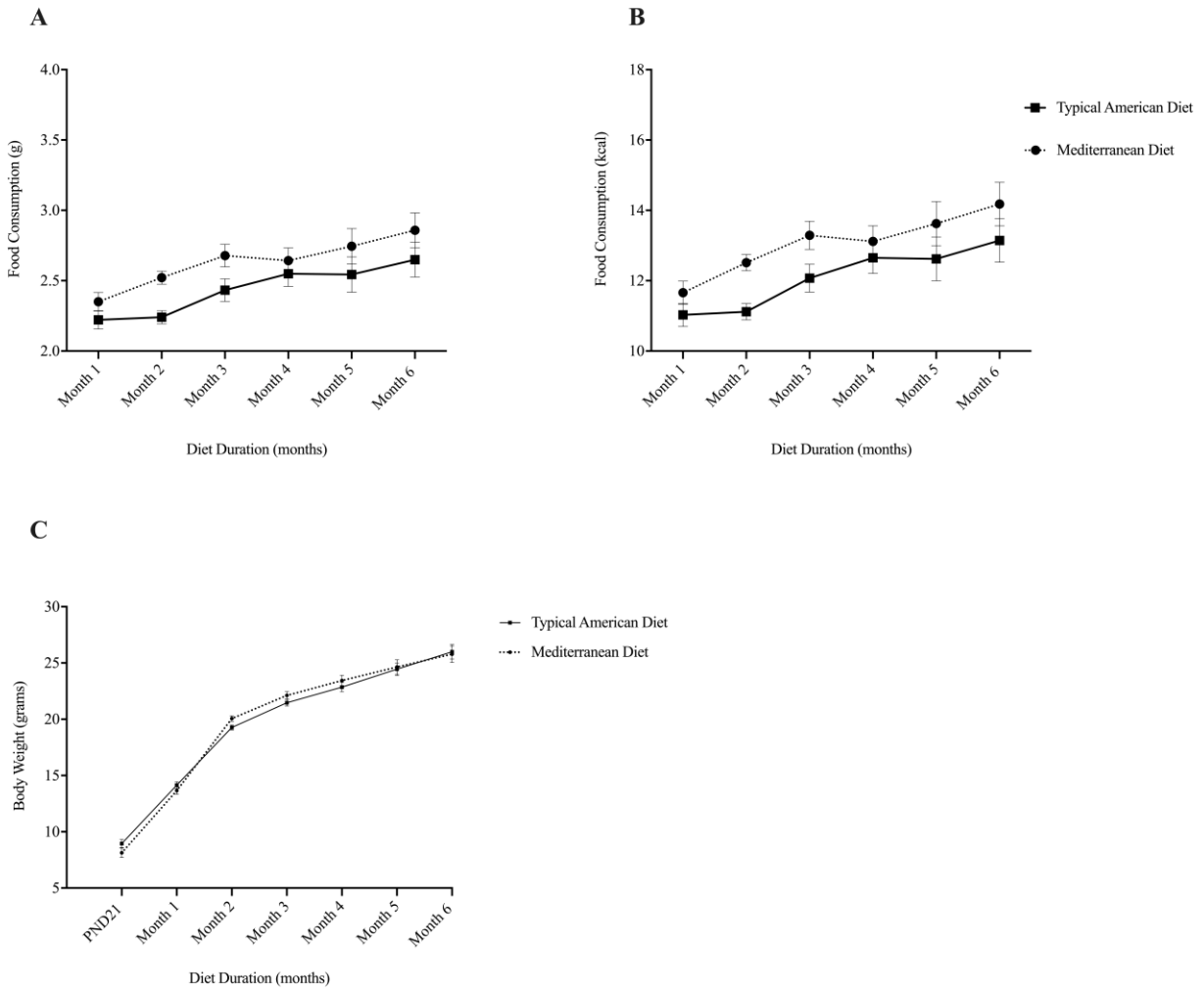


Figure 8. Food Consumption and Body Weight Throughout Diet Administration in Females. (A) A mixed-design ANOVA did not reveal a significant main effect of diet condition (MD vs. TAD) on food consumption in female mice, $p \geq 0.05$, N's =4. (B) There was no difference in kcal consumption between diet conditions, $p \geq 0.05$, N's = 4. (C) There was no significant main effect of diet condition on body weight between females on the MD and the TAD, $p \geq 0.05$, N's = 15–20. Bars represent ± SEM. Significant differences ($p < 0.05$) are designated by *.

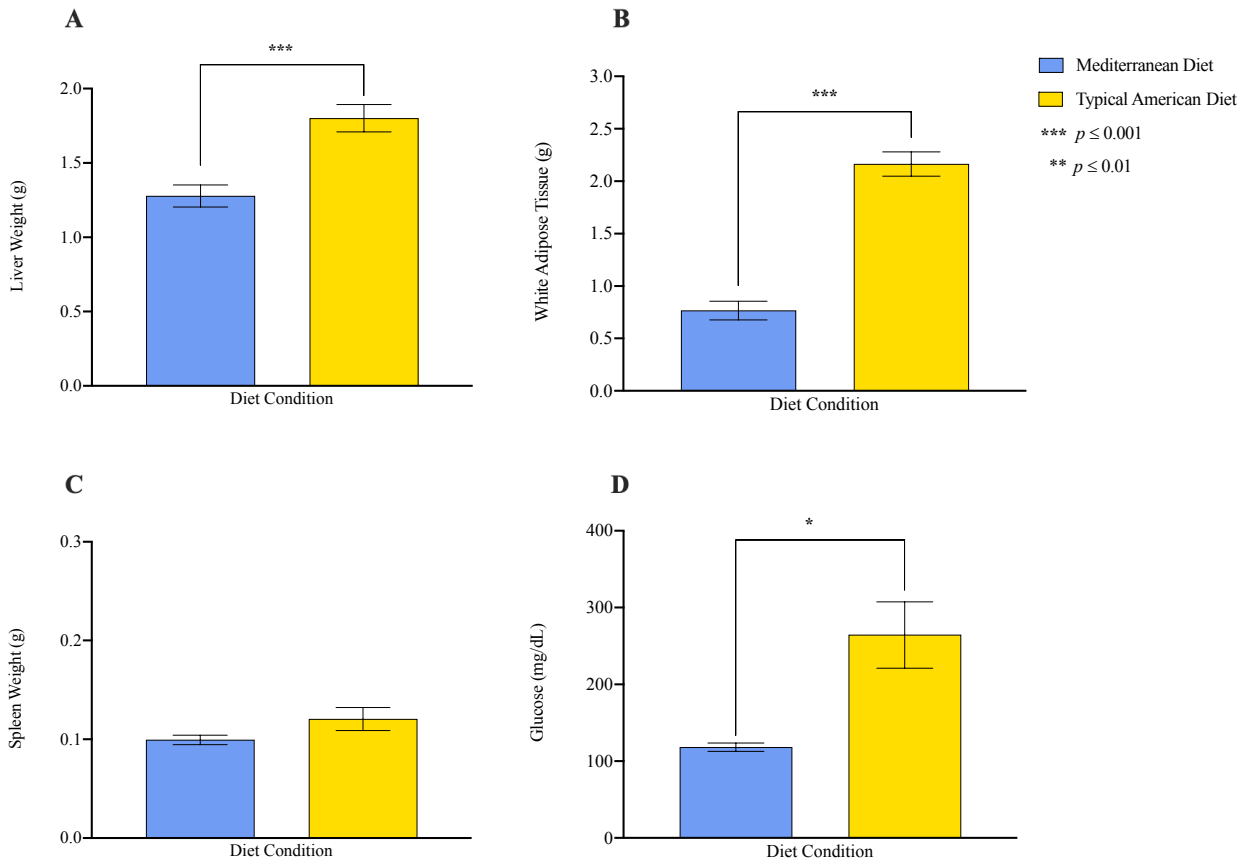


Figure 9. Organ and White Adipose Tissue Weight & Blood Glucose in Male Mice. (A) Male mice on the TAD had significantly heavier livers than those on the MD, $p \leq 0.001$, N's = 13–16. (B) Mice on the TAD had significantly more white adipose tissue (g) than those on the MD, $p \leq 0.001$, N's = 13–16. (C) There was no significant difference in spleen weight between diet conditions, $p \geq 0.05$, N's = 16–19. (D) Mice on the TAD had significantly higher blood glucose (mg/dL) than mice on the MD, $p \leq 0.01$, N's = 5–6. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *.

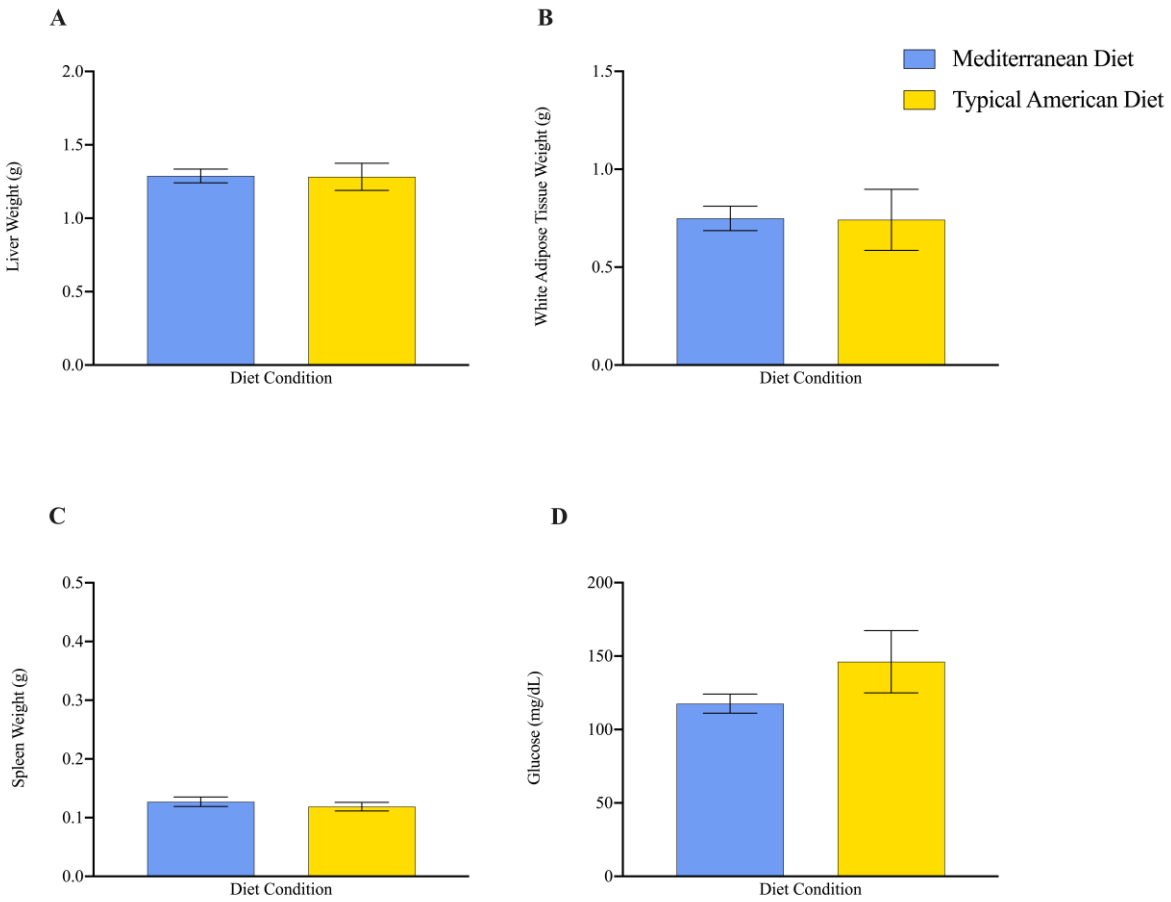
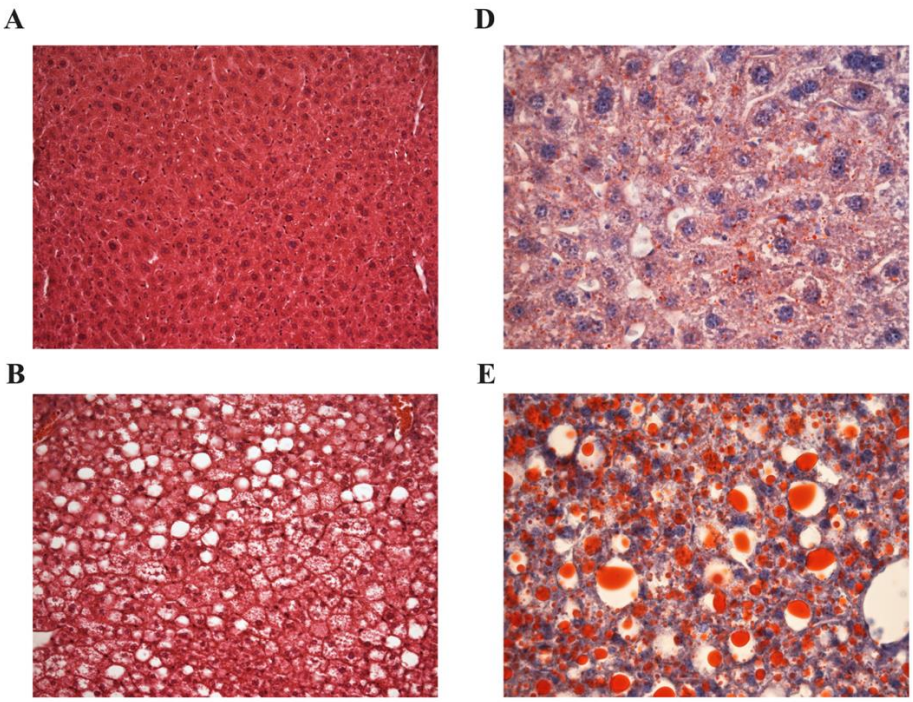


Figure 10. Organ and White Adipose Tissue Weight & Blood Glucose in Female Mice. There was no significant difference in weight between female mice on the MD and the TAD in the following tissues: (A) liver, (B) white adipose tissue, or (C) spleen, *p*s ≥ 0.05, N's =14–15. (D) Additionally, there was no significant difference in blood glucose (mg/dL) levels between female mice on the MD and TAD, $p \geq 0.05$, N's = 5–10. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *.



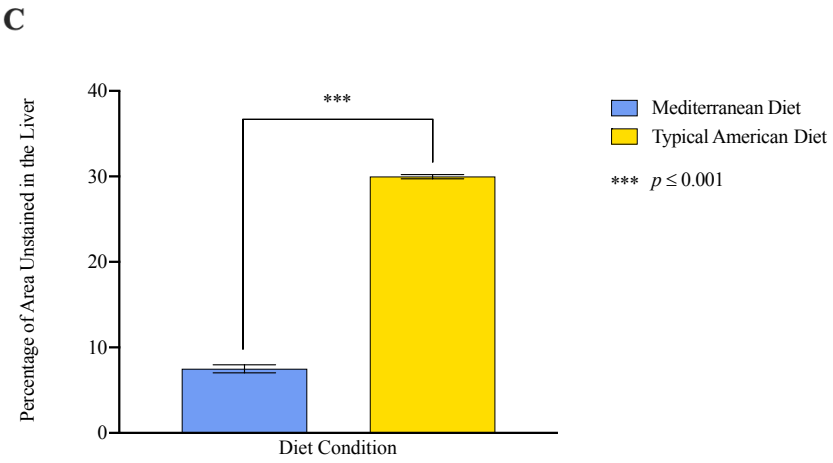
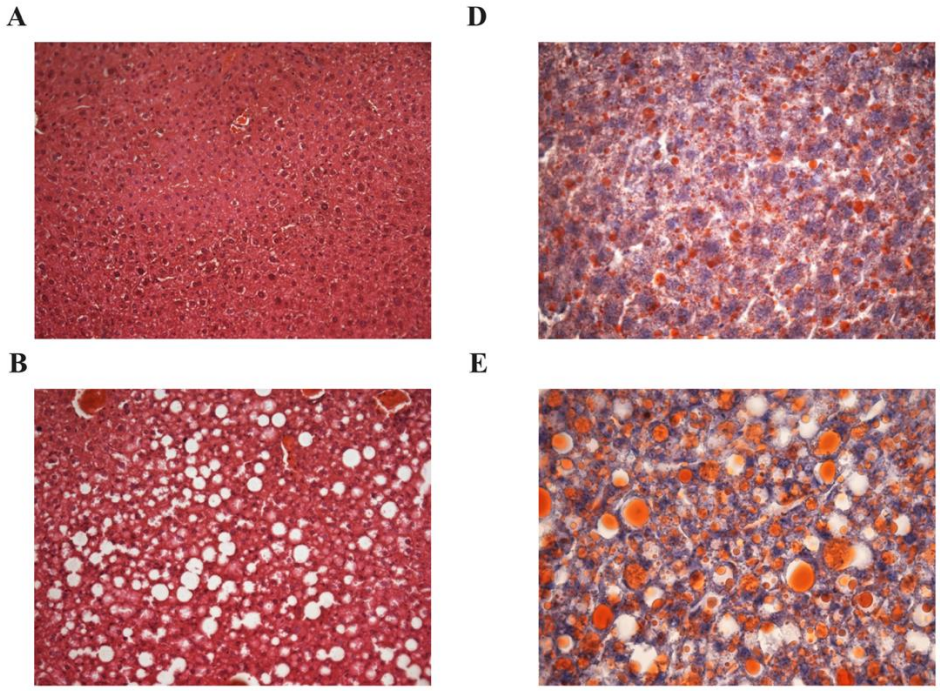


Figure 11. Liver Histology in Male Mice. (A) H & E-stained liver from a male mouse on the MD. (B) H & E-stained liver from a male mouse on the TAD. (C) An independent samples t-test revealed a significant difference in the percentage of pixelated area that was unstained following H & E staining, $p \leq 0.001$, N's = 11–12. (D) Oil-Red O-stained liver from a male mouse on the MD. (E)

Oil-Red O-stained liver from a male mouse on the TAD. (D–E) Lipids are stained red. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *.



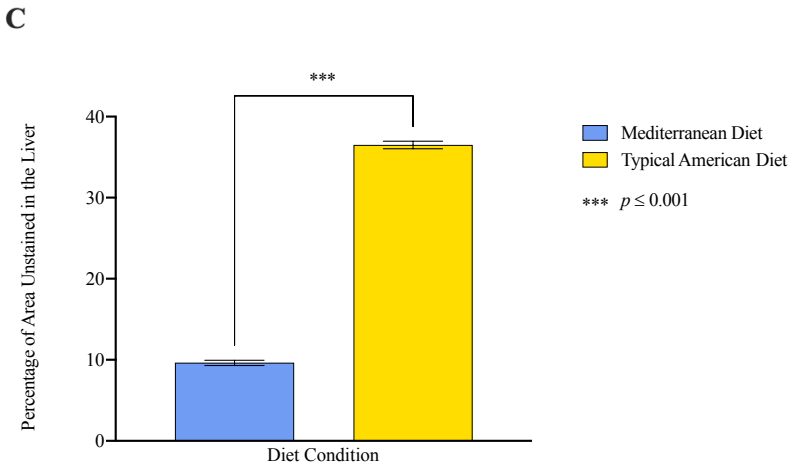


Figure 12. Liver Histology in Female Mice. (A) H & E-stained liver from a female mouse on the MD. (B) H & E-stained liver from a female mouse on the TAD. (C) An independent samples t-test revealed a significant difference in the percentage of pixelated area that was unstained following H &

E staining, $p \leq 0.001$, N's =12. (D) Oil-Red O-stained liver from a female mouse on the MD. (E) Oil-Red O-stained liver from a female mouse on the TAD. (D–E) Lipids are stained red. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

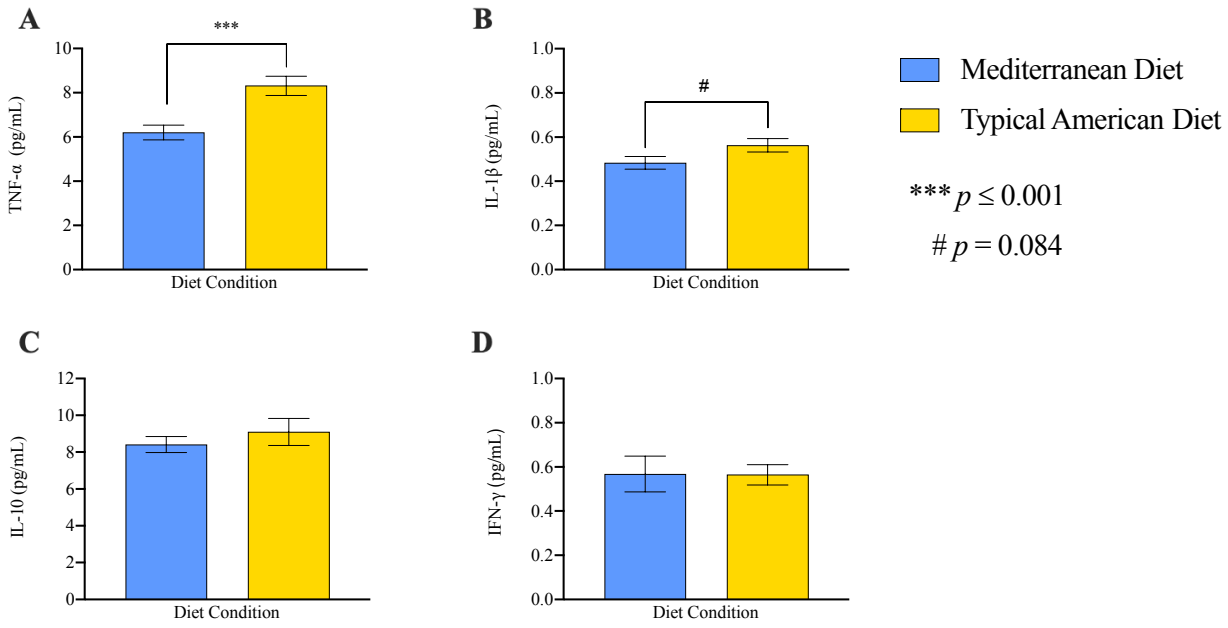


Figure 13. Peripheral Cytokine Production (pg/mL) in Serum of Male Mice. Independent samples t-tests were performed to compare the quantity of peripheral cytokines produced in serum following six months of diet. (A) Mice on the MD produced significantly less TNF-α in serum, compared to mice on the TAD, $p \leq 0.001$. (B–D) There was no significant difference in IL-1β, IL-10, or IFN- γ production between diet conditions, ps > 0.05. (A–D) N's = 11–16. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

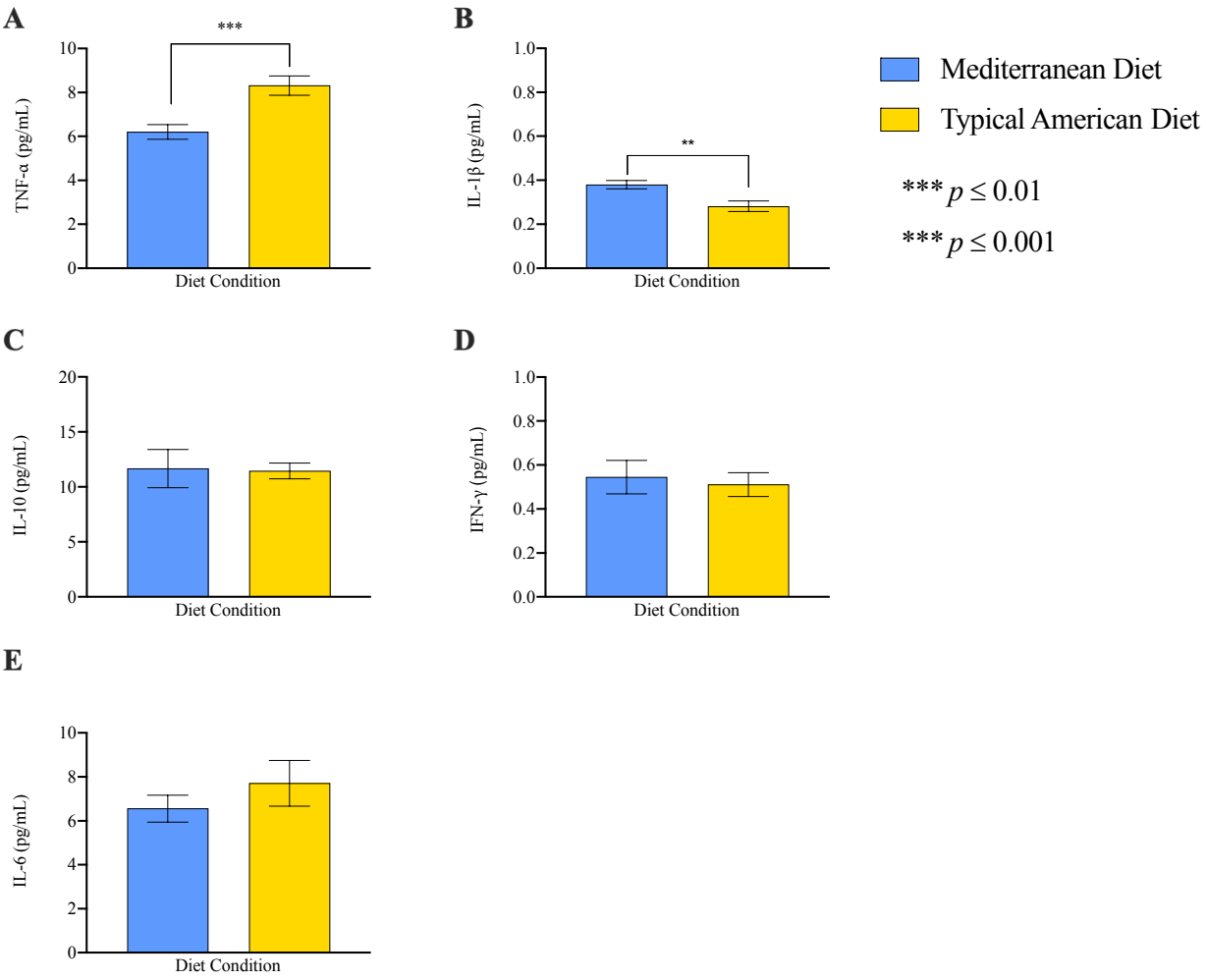


Figure 14. Peripheral Cytokine Production (pg/mL) in Serum of Female Mice. Independent samples t-tests were performed to compare the quantity of five cytokines produced in serum following six months of diet. (A) Female mice on the MD produced significantly less TNF-α in serum, in comparison to female mice on the TAD, $p \leq 0.001$, N's = 9–12. (B–D) There was no significant difference in IL-1β, $p > 0.05$, N's = 11–12, IL-10, $p > 0.05$, N's = 13, IFN- γ, $p > 0.05$, N's = 11–12, or IL-6 production between diet conditions, $p > 0.05$, N's = 13. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

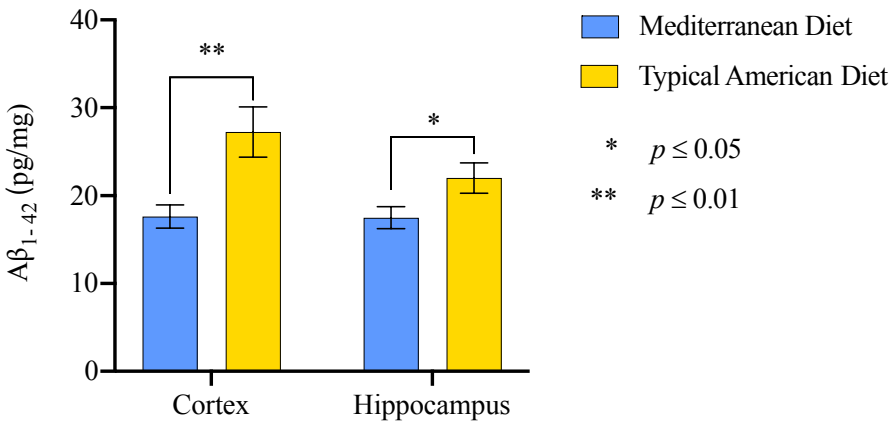


Figure 15. Soluble Aβ1–42 (pg/mg of protein) in the Cortex and Hippocampus of Male Mice.

Male mice on the TAD had significantly more soluble Aβ1–42 (pg/mg) in the cortex, $p \leq 0.01$, and in the hippocampus, $p \leq 0.05$, compared to those on the MD, N's = 12–16. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

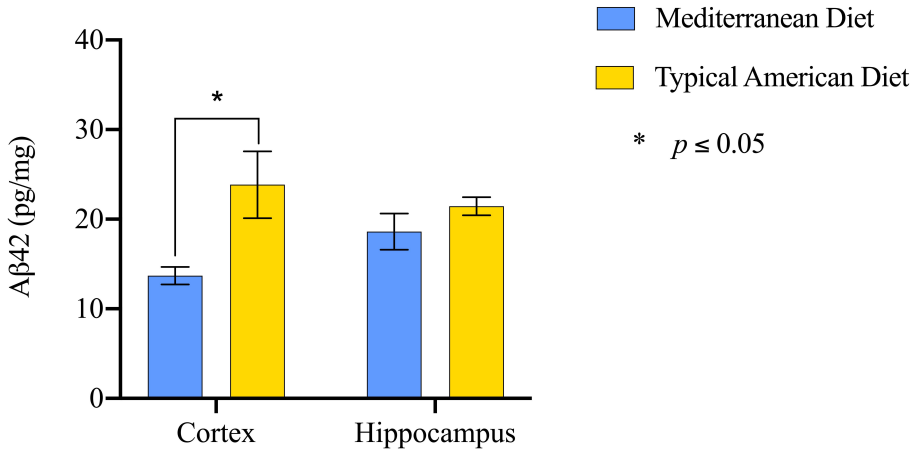


Figure 16. Soluble Aβ1–42 (pg/mg of protein) in the Cortex and Hippocampus of Female Mice.

Female mice on the TAD had significantly more soluble Aβ1–42 (pg/mg) in the cortex, $p \leq 0.05$, but not in the hippocampus, $p > 0.05$, compared to female mice on the MD, N's = 11–14. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

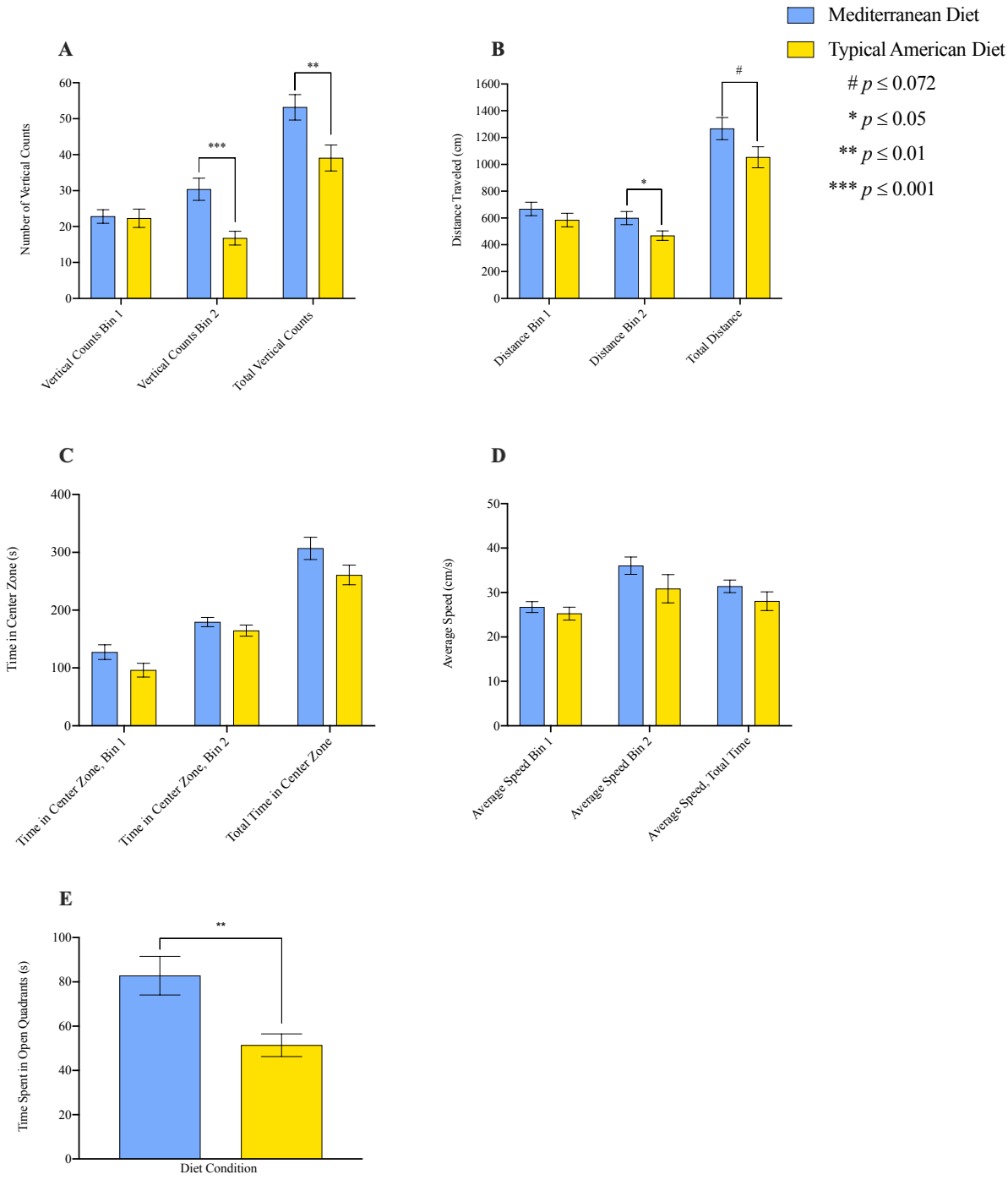


Figure 17. Open Field Test and Elevated Zero Maze Results in Male Mice. Multiple independent samples t-tests were performed to examine exploratory behavior, locomotor activity, and anxiety-like behavior. (A) Male mice on the MD exhibited significantly more vertical counts during the last five minutes of testing, and during the total testing time compared to mice on the

TAD, *p*s ≤ 0.01. (B) Mice on the MD traveled a significantly farther distance (cm) than those on the TAD during the last five minutes of testing $p \leq 0.05$. The difference in total distance (cm) traveled approached significance, $p = 0.072$. (C) There was no significant difference in time (s) spent in the center zone of the open field between the two diet conditions, *p*s > 0.05. (D) There was no significant difference in the average speed (cm/s) between the two diet conditions, *p*s > 0.05. (E) Mice on the MD spent significantly more time in the open quadrants of the elevated zero plus maze in comparison to mice on the TAD, $p \leq 0.01$. (A–E) N's = 13–20. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

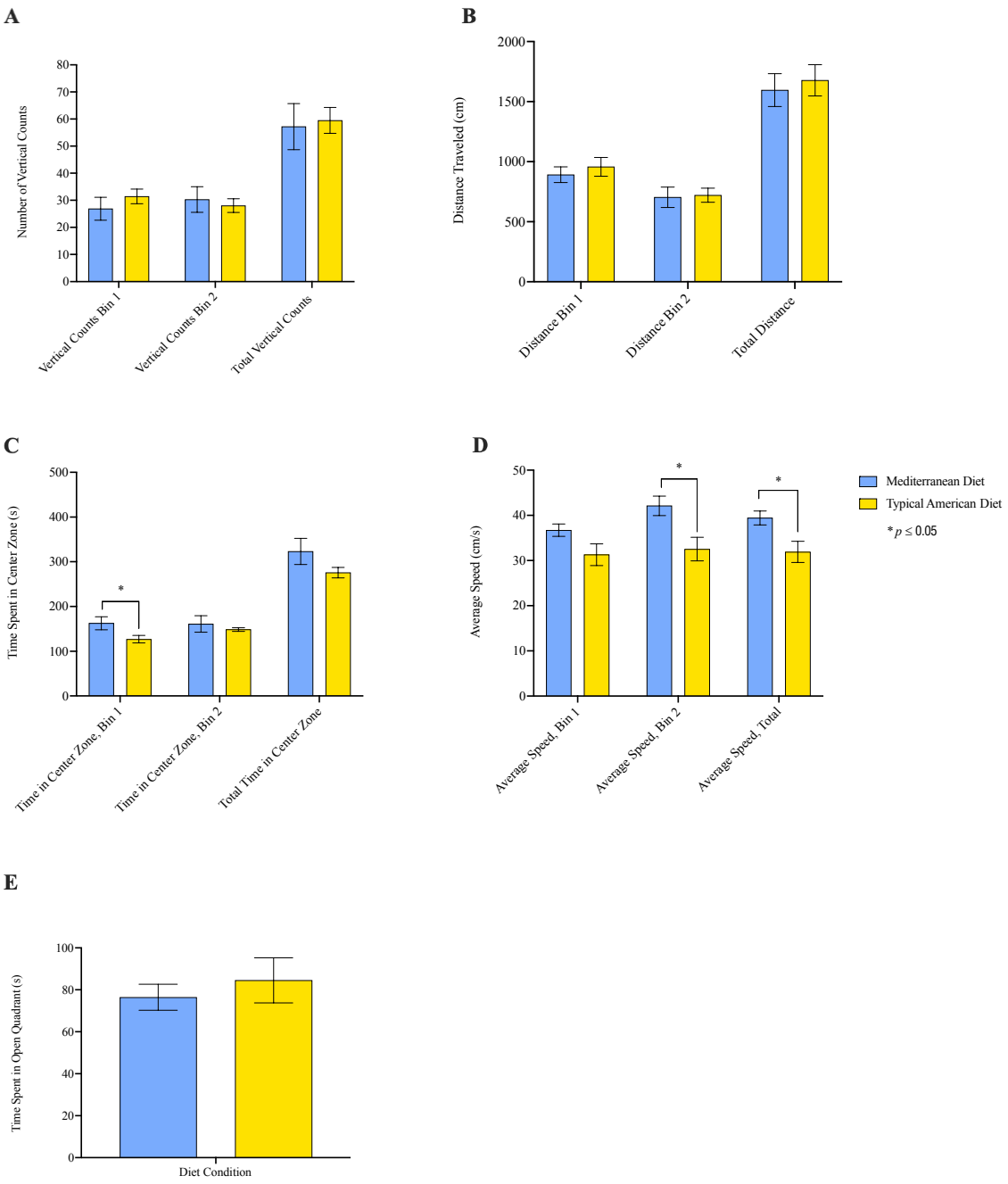


Figure 18. Open Field Test and Elevated Zero Maze Results in Female Mice. (A–B) The results revealed that there was no significant difference in vertical counts or distance traveled between female mice on the MD and TAD, *p*s > 0.05. (C) Female mice on the MD spent significantly more time in the center zone of the open field during the first five minutes of testing (bin 1), $p \leq 0.05$, but not in the last five minutes of testing (bin 2), or during the total testing time (10 minutes), *p*s > 0.05.

(D) There was no significant difference in the average speed (cm/s) between female mice on the MD and TAD during the first five minutes of testing (bin 1), $p > 0.05$. However, female mice on the MD were significantly faster during the last five minutes of testing, and throughout the total duration of testing (10 minutes), ps ≤ 0.05. (E) There was no significance difference in the amount of time spent in the open quadrants of the elevated zero maze between female mice on the MD and TAD, $p > 0.05$. (A–E) N's = 9–17. Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

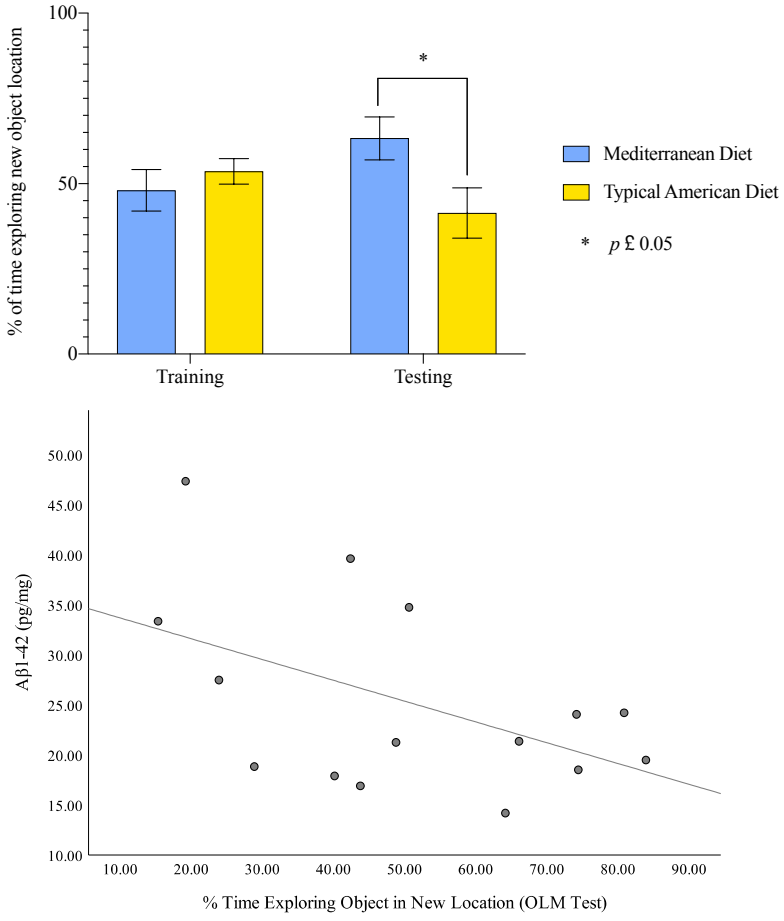


Figure 19. Spatial Memory in OLM Test in Male Mice. (A) Male mice on the MD spent significantly more time exploring the object moved to the novel location, compared to mice on the TAD, during testing, $p = 0.037$, N's = 10. Significant differences ($p \leq 0.05$) are designated by *. Bars

represent mean ± SEM. (B) **Mean of Aβ$_{1–42}$ in the Cortex by the Percentage of Time Exploring New Location Object in OLM in Male Mice.** There was a medium, negative correlation between the two variables, r = -.498, n = 15, p = 0.059, *ns*.

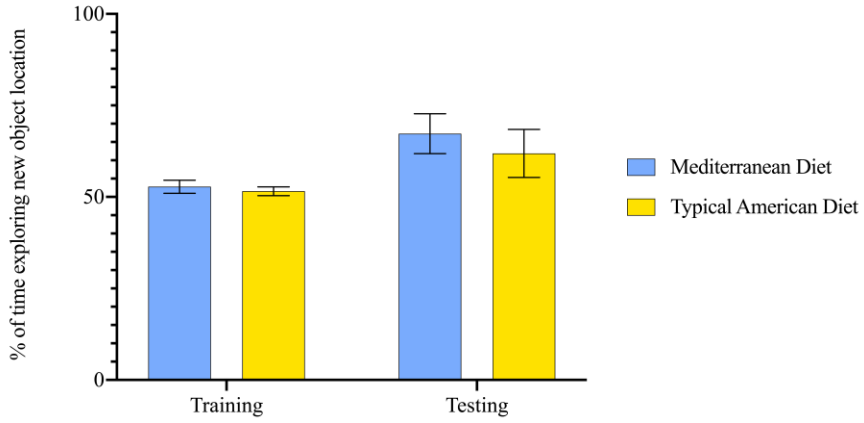


Figure 20. Diet Does Not Affect Spatial Memory in Female Mice. There was no significant difference in object exploration in the novel location between female mice on the MD and TAD, $p > 0.05$. Bars represent mean ± SEM.

5. Discussion for Experiment 1

The current experiment aimed to explore how early-life implementation and continued long-term adherence to the MD, could potentially prevent the development of AD pathologies and memory impairment later in adulthood, when compared to the TAD. Although there is abundant evidence that the MD is associated with protection against AD pathologies, such as Aβ accumulation in older adults, there is limited research on the underlying biological and behavioral mechanisms of a long-term, comprehensive MD, and how it potentially diminishes AD susceptibly in animal models (Berti et al., 2018; Rainey-Smith et al., 2018). Although numerous studies have explored the effects of supplementation of Mediterranean foods, like olive oil and fish oil, with standard chow rodent diets, these are not representative of a human MD. Based on the current available evidence,

the combined effects of multiple Mediterranean dietary components may be more protective against AD pathologies compared to supplementation of just one or two MD components (Szczechowiak et al., 2019). MD experts infer that foods and nutrients in the MD work synergistically to provide health benefits, such as protection against cognitive decline in elderly people (Trichopoulou et al., 2015; Trichopoulou et al., 2014).

Likewise, limited studies have examined the effects of a comprehensive TAD on AD biomarkers, behavior, and cognition in mice, as many prior experiments have utilized Western rodent diets with exaggerated fat (e.g., SFA) or sugar content that is not truly representative of the average American's diet. Commonly used Western rodent diets do not typically mimic multiple key variables of a TAD, such as average macronutrient densities, percentage of kilocalories from different sources of fat (SFA, MUFAS, and PUFAS), or *n*-6 to *n-3* ratio. Therefore, we addressed these limitations by investigating the long-term effects of two, *comprehensive*, macronutrient-matched diets (MD vs. TAD) on AD susceptibility in male and female C57BL/6 mice. The two experimental diets were designed to (1) mimic the average macronutrient density ranges found in human Mediterranean or American diets, (2) incorporate diverse food sources, rather than supplementation of one or two dietary components (3) control for energy availability between both diets.

To our knowledge, this is the first experiment to directly compare the effects of two macronutrient-matched rodent diets that mimic *several* key variables of either a typical Mediterranean or American diet on physiology, AD biomarkers, behavior, and cognition in wildtype mice. However, a similar experimental design was recently utilized by Shively and colleagues, in which experimenters created two diets resembling the MD or WD for nonhuman primates (Johnson et al., 2022; Johnson et al., 2021; C. A. Shively et al., 2020; Shively et al., 2019; Carol A. Shively et al., 2020). Experimenters found that long-term consumption of a WD

induced excess body fat, insulin resistance, and hepatosteatosis (Shively et al., 2019), anxiety (Johnson et al., 2022; Johnson et al., 2021), and the expression of pro-inflammatory genes (Johnson et al., 2021) compared to the MD in nonhuman primates.

The results of the current study revealed that a comprehensive MD protected male mice from developing biological, behavioral, and cognitive measures associated with AD, compared to the typical dietary pattern in the U.S. The current data lend support to prior research that has also revealed the harmful effects of high-fat, WDs on physiological, biological, behavioral, and cognitive markers associated with AD in male wildtype mice (Baranowski, Bott, & MacPherson, 2018; Gabriel et al., 2020; Kanoski et al., 2007; Pistell et al., 2010; Rutkowsky et al., 2018). However, the current findings addressed a gap in the literature and revealed that a TAD with a *lower* fat and sugar content compared to widely used WDs still provoked AD biomarkers, behavioral alterations, and spatial memory impairment in male mice. This further corroborates the hypothesis that the *typical* dietary pattern in America is linked to increased AD susceptibility in wildtype mice.

Interestingly, although both diets in the current study were macronutrient-matched, male mice assigned to the MD consumed more kilocalories per day than those assigned to the TAD. These results are not in line with our hypothesis that male mice on the MD or TAD would consume the same amount of food. A previous study investigating the effects of the WD found that male C57BL/6 consumed less food when placed on a WD, in comparison to a low-fat chow diet, most likely due to higher kilocalorie content (Casimiro et al., 2021). However, contrary to most prior research, the two diets utilized in the current study were macronutrient-matched, and thus provided the same amount of energy availability. Notably, although male mice on the MD consumed more total calories, they weighed less than male mice on the TAD, following six months of diet administration. Although prior research has clearly established that a high-fat WD (providing 40–

60 % kcal from fat) induces excess weight gain compared to low-fat diets (Graham et al., 2016; Heyward et al., 2012; Pistell et al., 2010), the current study is one of the first to illustrate that the TAD induces significant weight gain in male C57BL/6 mice.

Although the TAD currently utilized provided approximately 5–25% less kilocalories from fat than typical WDs used in research with C57BL/6 mice (Denver et al., 2018; Guillemot-Legris, Masquelier, et al., 2016; Heyward et al., 2012; Julien et al., 2010; Liu et al., 2014; Moser et al., 2018; Pistell et al., 2010; Rutkowsky et al., 2018), it still increased body weight compared to the MD in male mice. Similar to the current study, Graham and colleagues (2016) designed an animal fat and protein-based that was representative of the average diet in Western societies, and compared it to a plant-based, low-fat diet in male B6.APBTg and wildtype mice. Although the diets utilized by Graham and colleagues (2016) were not macronutrient-matched like the current study, the diets provided similar calorie content, and they induced significant weight gain in both B6.APBTg and wildtype mice following eight months of diet administration. Together, the current study and the previously described experiment (Graham et al., 2016) demonstrate that the *average* American diet engenders increased weight gain in male C57BL/6 mice. However, there were no differences in food consumption patterns or body weight in female mice. Notably, other researchers have also discovered a sexual dimorphism in feeding behavior and body weight in C57BL/6 mice. A recent study reported that male C57BL/6 mice are more susceptible to increased weight gain and insulin resistance from Western rodent diets compared to females (Casimiro et al., 2021).

Further, olive oil rich diets, with a high MUFA to SFA ratio, have been shown to protect rodents from excess body weight gain in comparison to SFA rich diets(Lauretti et al., 2017; Nakajima et al., 2020). However, even when examining Mediterranean dietary factors, there are conflicting results in the literature, and other studies have not found significant effects of such

factors on body weight in wildtype mice and transgenic mouse models of AD (Marchlewicz et al., 2022; Nardiello et al., 2018; Sharman et al., 2019). For example, Nardiello et al. (2018) found that an olive oil supplemented rodent diet did not affect body weight in wildtype mice, or a transgenic mouse model of AD. However, the previously discussed studies differ from the current study because they examined the effects of a standard chow diet supplemented with or without olive oil, rather than a comprehensive diet.

Overall, data from the current study suggest that the *type* of diet ingredients (e.g., butter and beef fat vs. olive oil and fish oil) could play a crucial role in the prevention of weight gain in male mice. As mid-life obesity is the number one modifiable risk factor for AD (Livingston et al., 2020), these data provide more insight on the potential use of the MD for weight gain prevention, compared to the TAD. Further, although the MD and TAD both provided the same percentage of kilocalories from fat, male mice assigned to the TAD had more abdominal white adipose fat and heavier livers compared to those on the MD, whereas there were no differences in females. This is supported by previous experiments that have also demonstrated that Western rodent diets increase white adipose fat in male C57BL/6 mice (Illesca et al., 2019) and 5XFAD mice (Lin et al., 2016). Notably, Illesca and colleagues (2019) found that hydroxytyrosol (a polyphenol in olive oil) mitigated metabolic impairment induced by a high-fat diet on white adipose tissue via NF-κB and Nrf2 pathways in male C57BL/6 mice.

We also explored the effects of diet on fat deposition in the liver, as nonalcoholic fatty liver disease (NAFLD) has been associated with increased risk of AD (Jeong et al., 2022) and cognitive decline in human subjects (Seo et al., 2016). As previously discussed, new evidence has also demonstrated that partial blockage of blood flowing to the liver impairs the liver's ability to clear $A\beta_{1-40}$and $A\beta_{1-42}$ in the periphery of the body, and thus exacerbates central $A\beta_{1-}$

$_{40}$and A$\beta_{1–42}$ in the brain in APP/PS1 mice (Cheng et al., 2023). Thus, we explored the effects of the MD and TAD on fat deposition in the liver, and histological results revealed that the TAD induced more fat deposition in the liver compared to the MD in both male and female mice. These data suggest that both the *type* of fat, the percentage of kilocalories from different sources of fat, and the *n*-6 to *n*-3 ratio is crucial for liver health.

Likewise, prior studies have also shown a WD, providing high SFA and *n*-6 to *n*-3 ratio, provoke excess fat deposition in the liver of wildtype mice (Kim et al., 2016; Li et al., 2018), and induce NAFLD and exacerbate A$\beta_{1–42}$ plaque burden in APP-Tg mice (Kim et al., 2016). Additionally, a recent study found that long-term WD consumption increased hepatosteatosis compared to a macronutrient-matched MD in female, non-human primates (Shively et al., 2019). These results are further supported by previous research that explored the effects of Mediterranean dietary factors on liver health in human subjects (George et al., 2022; Ryan et al., 2013). For example, George and colleagues (2022) utilized data from the ATTICA study and found that each one unit increase of the Mediterranean diet score was associated with 17% less risk of NAFLD in healthy adults. Similar to the MD utilized in the current study, a plant-based diet with a balanced *n*-6 to *n*-3 ratio protected female C57BL/6 mice from fatty liver disease compared to a WD (Li et al., 2018). Further, a recent study demonstrated that the combined effects of a Mediterranean fat blend reduced lipid peroxidation and ROS provoked by high-fat diet induced steatohepatitis in primary mouse hepatocytes (Castellanos-Tapia et al., 2020). These data further support the current findings that the combined effects of multiple MD factors could protect C57BL/6 mice from fatty liver disease compared to the TAD. As NAFLD has been linked to increased AD risk, evidence presented in prior research and the current study indicates that plant-based or MD-style diets prevent fat deposition in the liver, while a TAD induces fatty liver in male and female C57BL/6 mice.

In addition to increased fat deposition in the liver, the TAD also increased serum TNF-α in male and female mice, and only moderately increased serum IL-1β in male mice compared to mice on the MD. These results are supported by previous findings that a WD increases pro-inflammatory cytokine production in C57BL/6 mice (Li et al., 2018; Pistell et al., 2010). However, we did not see significant differences in other pro-inflammatory markers, like IFN- γ, in male mice, as the TAD utilized in the current study had lower fat content compared to commonly used WDs.

The current findings are also supported by previous experiments that demonstrated the anti-inflammatory capacity of Mediterranean dietary factors in C57BL/6 mice (Akerele & Cheema, 2018; Illesca et al., 2019; Li et al., 2018). Although there is limited research on the potential, anti-inflammatory effects of a complete MD in mice, prior studies demonstrate that individual elements in the MD are anti-inflammatory. For example, prior studies have shown that a plant-based diet (Li et al., 2018) or a Mediterranean fatty acid diet (Wenzel et al., 2022) reduces inflammation in female mice. Interestingly, colon length is also a known indicator of inflammation, and increased inflammation in the colon leads to shrinkage (Piazzi et al., 2019; Suzuki et al., 2005). In the current study, male mice on the MD had significantly longer colons in comparison to the male mice on the TAD. Likewise, Piazzi and colleagues (2019) recently reported that a Mediterranean rodent diet, supplemented with multiple plant polyphenols, protected a mouse model of colon cancer from colon shrinkage. Thus, the current results, in addition to other studies (Piazzi et al., 2019) suggest that the MD could potentially protect mice from inflammation in the colon. Overall, the results of the current study fill a gap in this literature and demonstrate that a comprehensive MD protects against the elevation of serum TNF-α compared to the TAD in both male and female mice. However, our hypothesis that the MD would prevent elevated levels of serum IL-1β, IL-10, or IFN- γ was not confirmed, and more

research is needed to understand the complex relationship between the MD and innate immune response in C57BL/6 mice.

The results of the current study also revealed that the TAD provoked an increase in $A\beta_{1-42}$ in the cortex of both male and female mice, and in the hippocampus of male mice, compared to the MD. In support of these findings, prior studies have demonstrated that a WD induces $A\beta_{1-42}$ in C57BL/6 mice (Moser et al., 2018), although few have explored the effects of a TAD on AD susceptibility in wildtype mice, like the current study. Nevertheless, Western diets designed for rodents have also been shown to exacerbate the progression of AD pathologies in transgenic mouse models of AD (Graham et al., 2016; Ho et al., 2004; Hooijmans et al., 2007; Julien et al., 2010; Vandal et al., 2014; Walker et al., 2017). Like the current study, Graham and colleagues (2016) created a rodent diet that resembled the average diet consumed in the U.S. Interestingly, they found that the WD exacerbated $A\beta_{1-42}$ plaque burden in the hippocampus, but not the cortex of male B6.APBTg mice. The current results are also supported by prior studies that also discovered the amyloid reducing capacity of MD components, like olive oil and fish oil. For example, prior research has found that standard chow diets supplemented with olive oil reduce or prevent hippocampal and cortical $A\beta$ in transgenic mouse models of AD (Lauretti et al., 2017; Qosa et al., 2015), and wildtype mice (Abuznait et al., 2013), respectively. Additionally, previous studies have demonstrated that plant polyphenols in olive oil can be used as amyloid reducing agents in rats (Luccarini et al., 2014) and mice (Grossi et al., 2013; Nardiello et al., 2018). Similarly, fish oil and DHA supplementation has been shown to mitigate hippocampal and cortical $A\beta$ production in transgenic mice (Green et al., 2007; Lim et al., 2005; Oksman et al., 2006; Perez et al., 2010; Zhou et al., 2018). Collectively, these data demonstrate that

Mediterranean dietary factors, particularly olive oil and fish oil, could potentially be utilized as amyloid lowering therapies.

Overall, the results of the current study suggest that the combined effects of Mediterranean macronutrient sources, such as the types of carbohydrates, protein, and fat, in addition to both insoluble and soluble fiber, work together to prevent elevated $A\beta_{1-42}$ production in the cortex and hippocampus, compared to the TAD in male mice. Based on our current findings in both liver and brain tissue, we infer that the TAD induced fatty liver disease and impaired the liver's ability to clear circulating $A\beta_{1-42}$ in the blood. Cheng and colleagues (2023) recently demonstrated that blocking blood flow to the liver increased circulating Aβ in mice, and thus, we infer that impaired peripheral $A\beta_{1-42}$ clearance potentially increased $A\beta_{1-42}$ transport to the brain in male mice on the TAD. We were particularly interested in exploring the relationships between abdominal white adipose fat weight, liver weight, serum TNF-alpha, and $A\beta_{1-42}$, as increased adiposity has been associated with inflammation and AD biomarkers. We found that white adipose fat strongly correlated with cortical $A\beta_{1-42}$ and serum TNF- α, while serum TNF- α strongly correlated with both liver and body weight. These relationships indicate that increased fat deposition in the abdomen and liver is associated with increased TNF-α production. Similar to the current findings, Her and colleagues (2020), demonstrated that NAFLD induced chronic inflammation in mice (Her et al., 2020). Likewise, a recently published meta-analysis revealed that NAFLD was associated with serum TNF-α in adults (Duan et al., 2022).

In addition to inducing fatty liver and TNF-α, we hypothesize that the TAD potentially disrupted the BBB, and increased the ability of peripheral $A\beta_{1-42}$ to enter the brain via the RAGE receptor. Future studies should investigate the relationship between the liver and brain, to understand if the TAD provokes both peripheral $A\beta_{1-42}$, and brain generated $A\beta_{1-42}$ via de novo

synthesis. Overall, more research is needed to explore the relationships between liver function, inflammation, and peripheral Aβ transport to the brain following MD or TAD administration, as the liver-brain axis could be a key target for AD therapeutics.

The results of behavioral testing revealed that the TAD altered locomotor activity in both male and female mice. Male mice on the TAD traveled less distance within the open field chamber compared to male mice on the MD. However, in female mice, there were no differences in the distance traveled, although female mice on the MD traveled faster than female mice on the TAD. Our hypotheses were only partially supported, because the TAD decreased exploratory activity and increased anxiety-like behavior compared to the MD in male mice, but not female mice. Interestingly, prior studies have also observed sexual dimorphisms in behavior between male and female C57BL/6 mice following exposure to a high-fat WD (Casimiro et al., 2021). Casimiro and colleagues found that a high-fat WD decreased locomotor activity in male, but not female C57BL/6 mice. Like the current results, other studies have demonstrated that olive oil or fish oil supplementation increases locomotor activity in male rats (Nakajima et al., 2020) and mice (Oksman et al., 2006; Walker et al., 2017).

Additionally, the MD protected male mice from spatial memory impairment in comparison to the TAD in the OLM task. During the training sessions of OLM, mice spent the same amount of time exploring the two identical objects, and during testing, mice on the MD spent more time exploring the object that was moved to the novel location, compared to those on the TAD. Prior studies have clearly established that Western rodent diets impair learning and memory processes (Abbott et al., 2019; Kanoski & Davidson, 2011). The current results are supported by prior studies that have revealed the harmful effects of Western rodent diets (providing about 40–60% kcal from fat) on spatial learning and memory, specifically in OLM task (Heyward et al., 2012),

novel object recognition task (NOR) (Denver et al., 2018; Heyward et al., 2012), T-maze (Arnold et al., 2014; Pistell et al., 2010), and Morris water maze (Denver et al., 2018; Xu et al., 2018), in mice. Additionally, numerous studies have clearly demonstrated that a WD specifically impairs spatial learning and memory in rats (Cordner & Tamashiro, 2015; Kanoski & Davidson, 2011; Kanoski et al., 2007; Molteni et al., 2002; Stranahan et al., 2008).

Moreover, the results of the current study are supported by previous findings on the protective capacity of plant-based diets (Li et al., 2018), and diets supplemented with plant polyphenols (Grossi et al., 2013; Nardiello et al., 2018; Pérez-Cañamás et al., 2016), or olive oil (Farr et al., 2012; Lauretti et al., 2017) on learning and memory in mice. For example, hydroxytyrosol, a prominent plant polyphenol in olive oil, improved spatial memory and object discrimination in a transgenic mouse model of AD (Nardiello et al., 2018). Lending support to these findings, recent studies have found that the combined effects of multiple dietary components in the MD support cognition in mice (Li et al., 2018; Sharman et al., 2019). For example, Li and colleagues (2018) examined the effects of a PUFA-rich, plant-based diet versus a WD in female C57BL/6 mice. Mice were administered one of the two experimental diets for three months prior to behavioral testing, and the results revealed that the plant-based diet significantly enhanced spatial learning in comparison to the WD (Li et al., 2018). Additional research has also shown that short-chain fatty acids, produced by gut microbiota following fiber intake, protect cognition. For example, sodium butyrate has been shown to improve learning and memory in a transgenic mouse model of AD (Fernando et al., 2020). As fiber was a key component in the MD utilized in the current study, the current results support previous findings on the benefits of high-fiber diets on learning and memory in mice.

Further analysis of the data also revealed that higher levels of cortical $A\beta_{1-42}$ were moderately, but not significantly, correlated with reduced object exploration of the novel location during OLM testing in male mice. The trend in this data suggests that the development of cortical $A\beta_{1-42}$, induced by the TAD, may disrupt spatial learning and memory in male C57BL/6 mice, compared to the MD, and further strengthens our hypothesis that the MD would hinder the development of AD neuropathology and cognitive impairment compared to the TAD.

CHAPTER 3: EXPERIMENT 2, THE PROTECTIVE EFFECTS OF THE MEDITERRANEAN DIET AGAINST LPS-INDUCED CENTRAL INFLAMMATION

Abbreviated Introduction

As previously discussed, WDs have been shown to provoke peripheral inflammation in wildtype mice (Graham et al., 2016; Li et al., 2018; Pistell et al., 2010). Likewise, the current study also found that the TAD increased serum TNF-α in male and female mice, and moderately increased serum IL-1β in male mice, compared to the MD. WDs have also been shown to induce or exacerbate central inflammation in the cortical (Liu et al., 2014; Pistell et al., 2010; Walker et al., 2017) and hippocampal regions (Liu et al., 2014) of wildtype mice (Liu et al., 2014; Pistell et al., 2010), and transgenic mouse models of AD (Graham et al., 2016; Walker et al., 2017), respectively. As chronic inflammation is connected to the neuropathology of AD, these data suggest that a WD may trigger or exacerbate AD neuropathology via inflammatory mechanisms.

Increased circulation of pro-inflammatory cytokines from peripheral organs and blood can induce the production of cytokines in the brain via transduction of the vagus nerve. Stimulation of vagal afferents activates the brain's resident immune cell, microglial cells, and microglia respond to inflammatory triggers by secreting neurotoxic factors, like pro-inflammatory cytokines and reactive oxygen species (Biesmans et al., 2013; Block et al., 2007; Dantzer, 2018; Steinberg et al., 2016; Zanos et al., 2018). As the current study observed an increase in serum TNF-α following consumption of the TAD, we also hypothesized that the TAD would induce increased gene expression of pro-inflammatory cytokines in the brain compared to the MD.

Accordingly, study two was conducted to further examine the effects of the TAD and MD, combined with an inflammatory stimulus, to investigate the potential, protective effects of the MD against external inflammatory triggers that could harm the CNS. As previously discussed, there are numerous environmental inflammatory triggers, such as allergies, pollution, pathogens, viruses, chronic stress, and unhealthy foods, that activate the innate immune response (Macdonald & Monteleone, 2005; Parham, 2013). Thus, bolstering the immune system with a healthy diet could potentially protect the body from inflammation induced by external sources.

As wildtype mice do not typically produce high levels of pro-inflammatory cytokines without an inflammatory stimulus, our laboratory utilizes lipopolysaccharide (LPS) to induce the innate immune response. LPS comes from the cell wall of gram-negative bacteria, such as *Escherichia coli* (*E. coli)*, and it is a toll-like receptor 4 (TLR4) agonist. As previously noted, toll-like receptors are pathogen recognition receptors that respond to inflammatory triggers by activating immune cells and inducing a signaling cascade to protect the body from unknown pathogens (Lee et al., 2003; Parham, 2013). The downstream effects following TLR4 activation turns on the transcription factor, NF-κB, and induces transcription of pro-inflammatory cytokine genes (Liu et al., 2017). Activation of this pathway has been shown to provoke the pro-inflammatory M1 phenotype of macrophages and microglia, and thus, chronic activation of NF-κB promotes microgliosis in the brain (Liu et al., 2017).

Numerous studies have utilized LPS to induce both peripheral and central inflammation in mice (Biesmans et al., 2013; Kahn et al., 2012; Kranjac et al., 2012; Lee et al., 2008; Weintraub et al., 2013; Zhao et al., 2019). Historically, our laboratory has demonstrated that seven consecutive days of intraperitoneal (i.p.) LPS (250μg/kg) injections induces soluble $A\beta_{1-42}$ in the hippocampus, and disrupts learning and memory in contextual fear conditioning (Kahn et

al., 2012; Weintraub et al., 2013). These findings are supported by other researchers who have also demonstrated that LPS treatment induces cognitive deficits in the MWM task (Sparkman et al., 2005; Zhao et al., 2019), and auditory and contextual fear conditioning (Pugh et al., 1998).

Our laboratory has repeatedly shown that one i.p. injection of LPS (250μg/kg) induces mRNA expression of pro-inflammatory cytokines and reduced mRNA expression of BDNF in the dorsal hippocampus of C57BL/6 mice (Kranjac et al., 2012). Specifically, the dorsal hippocampus has abundant cytokine receptors, and LPS treatment induces a more rapid inflammatory response in the dorsal hippocampus compared to ventral hippocampus (Onufriev et al., 2018; Parnet et al., 2002).

Prior studies have clearly demonstrated that IL1-β disrupts learning and memory in hippocampus-dependent tasks (Barrientos et al., 2002; Barrientos et al., 2004), and TNF-α interferes with synaptic transmission (Pickering et al., 2005). Prior research has further examined the relationship between cytokines and cognitive dysfunction, and demonstrated that LPS-induced inflammation decreases brain-derived neurotrophic factor (BDNF) (Kranjac et al., 2012; Schnydrig et al., 2007), a key player in memory consolidation and synaptic plasticity (Lee et al., 2004). BDNF loss is associated with several neurogenerative diseases, like AD (Miranda et al., 2019) and research has shown that AD patients have reduced levels of serum BDNF compared to healthy individuals (Gezen-Ak et al., 2013). Thus, BDNF is an AD biomarker that is commonly measured in addition to Aβ and BACE1.

Notably, multiple studies have shown that high SFA, Western rodent diets reduce BDNF in rats (Kanoski et al., 2007; Molteni et al., 2002; Stranahan et al., 2008) and mice (Pistell et al., 2010; Tozuka et al., 2010), while diets supplemented with plant polyphenols have been shown to protect mice from high-fat diet induced BDNF loss (Liu et al., 2014). Although there is little

research available on the effects of a comprehensive MD on BDNF, a prior study in human subjects found that high MD adherence was associated with higher levels of BDNF (Sánchez-Villegas et al., 2011). Based on the previously discussed findings, we aimed to further assess the relationship between diet and mRNA expression of cytokines, BDNF, and BACE1 in the dorsal hippocampus, to investigate the potential, protective effects of the MD against LPS-induced inflammation and AD biomarkers, compared to the TAD.

Hypotheses

Based on preceding evidence in the scientific literature, we hypothesized that early-life implementation and long-term consumption of a comprehensive MD would protect adult mice from LPS-induced mRNA expression of TNF-α, IL-1β, IL-6 in the dorsal hippocampus. Additionally, based on our findings in study one, in which the TAD generated significant $A\beta_{1–42}$ in the cortex of both male and female mice, and in the hippocampus of male mice compared to the MD, we hypothesized that mice on the TAD would also have increased mRNA expression of BACE1. BACE1 is an enzyme that is involved in the pathogenic cleavage of the membrane protein, amyloid precursor protein, a process that results in the formation of Aβ peptides (LaFerla et al., 2007; LaFerla & Oddo, 2005). Thus, we hypothesized that the TAD would increase BACE1 compared to the MD, particularly in male mice, as they had significant differences in $A\beta_{1–42}$ in the hippocampus, but the female mice did not. Further, as prior studies have shown that Western rodent diets promote mRNA expression of pro-inflammatory cytokines and reduce mRNA expression of BDNF and anti-inflammatory cytokines (Pistell et al., 2010), we also hypothesized that the MD would protect mice from BDNF and IL-10 loss following LPS treatment.

1. Materials and Methodology for Experiment 2

Experimental Design

Following 6 months and 21 days of diet administration (MD or TAD), male and female C57BL/6 mice were randomly assigned to one of two treatment conditions: one intraperitoneal (i.p.) injection of LPS (*E. Coli* serotype O26:B6; Sigma-Aldrich, St. Louis, MO) or sterile saline (Dulbecco's PBS; Caisson Laboratories, Smithfield, UT) at a dose of 250 µg/kg, four hours prior to tissue collection. Injections were performed between 0800 h and 1100 h. Our laboratory has previously demonstrated that one i.p. injection of LPS increases mRNA expression of IL1-β in both the cortex and the hippocampus of male C57BL/6 mice four hours following treatment (Kranjac et al., 2012). Thus, we utilized the same model performed by Kranjac and colleagues (2012) to induce central inflammation in the dorsal hippocampus following long-term diet administration. See Figure 21 for experimental timeline.

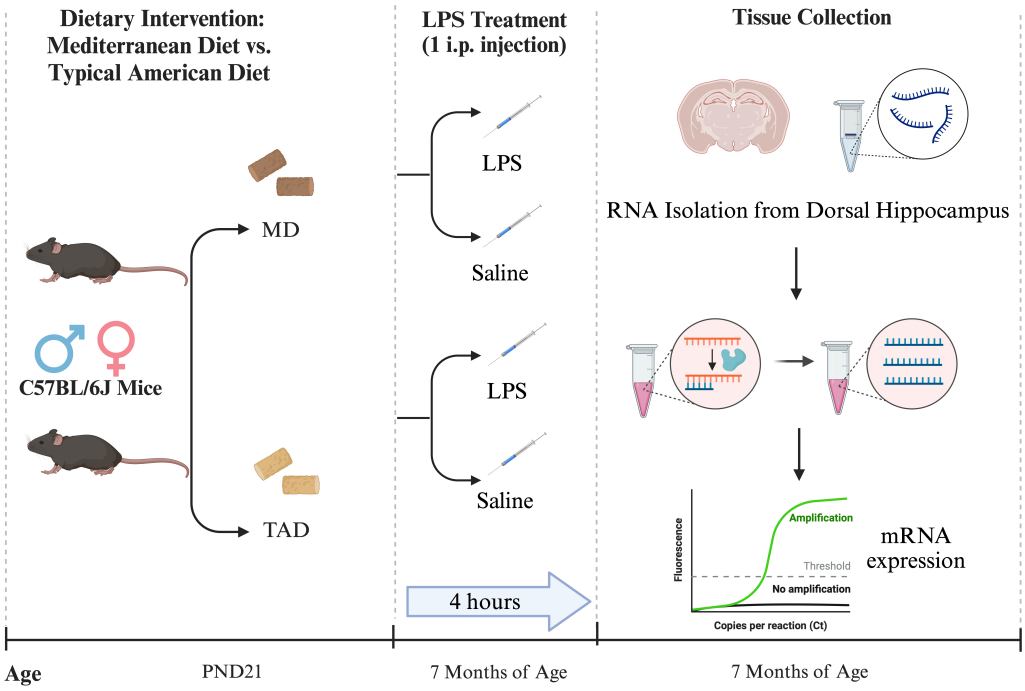


Figure 21. Experimental Timeline for Experiment 2.

Quantitative reverse-transcription polymerase chain reaction (qRTPCR)

Central gene expression for brain derived neurotrophic factor (BDNF), Beta-secretase 1 (BACE1), TNF-α, IL-1β, IL-10, IL-6, and β-actin (endogenous control gene) was measured in the dorsal hippocampus, utilizing quantitative reverse-transcription polymerase chain reaction (qRTPCR). First, mRNA was isolated from hippocampal tissue utilizing a Maxwell LEV simplyRNA Purification kit (Maxwell, Promega Corporation, Madison, WI) and measured for quantity and purity with a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, NanoDrop Products, Wilmington, DE). Subsequently, reverse transcription was performed utilizing Script Reverse Transcriptase Supermix (Bio-Rad, Hercules, CA) and the 7500 Real-Time PCR Thermal Cycling System (Applied Biosystems, Foster City, CA). The RT thermocycler settings were as follows: 5 min at 25 ºC, 30 min at 42 ºC, and 5 min at 85 ºC.

Expression levels of mRNA for the target genes were then measured using iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA) and PrimePCR Probe Assay probes (Bio-Rad, Hercules, CA). Samples were run in triplicate for each gene, and the relative amount of cDNA from the fluorescence data was compared and normalized to the relative amount of β-actin utilizing the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). PCR thermocycle settings for PCR were: 30 seconds at 95 ºC, 5 seconds at 95 ºC, and 39 cycles of 30 seconds at 60 ºC.

Statistical Analyses

Multiple analyses of variance (ANOVAs) were conducted utilizing Prism (GraphPad Software, Inc., San Diego, Ca), to examine the effects of diet condition (MD or TAD) and treatment condition (LPS or saline) on gene expression. An alpha level of $p < 0.05$ was considered significant. If a sample had a quantitation cycle (Cq) standard deviation of 0.250 or

higher, the outlier of the triplicates was removed from the dataset. If the Cq standard deviation was still more than 0.25 following this correction, the sample was excluded from data analysis.

3. Results

Gene Expression of BDNF and BACE1 in the Hippocampus of Male Mice.

A two-way ANOVA revealed a significant main effect of diet condition (MD or TAD) on hippocampal BDNF mRNA, $F(1, 38) = 9.263$, $p = 0.004$, in which mice on the MD had increased BDNF gene expression compared to those on the TAD. There was no significant main effect of injection (LPS or saline), $F(1, 38) = 0.069$, $p = 0.794$. Post-hoc analyses revealed that mice on the MD had increased BDNF gene expression compared to those on the TAD following LPS treatment, $p = 0.006$. However, a two-way ANOVA did not reveal a significant main effect of injection, $F(1,39) = 3.485$, $p = 0.07$, or diet condition, $F(1,39) = 2.606$, $p = 0.115$, on BACE1 gene expression in male mice. See Figure 22.

Gene Expression of TNF-α, IL-1β, IL-6, and IL-10 in the Hippocampus of Male Mice.

A two-way ANOVA revealed a significant main effect of injection on TNF-α gene expression, $F(1,38) = 73.55$, $p \leq 0.001$, such that male mice that received LPS treatment had increased TNF-α gene expression compared to mice that received sterile saline. There was also a significant main effect of diet condition, $F(1,38) = 4.320$, $p = 0.045$, such that mice on the TAD had increased TNF-α gene expression than those on the MD. This was further supported by a significant interaction, $F(1,38) = 4.242$, $p = 0.0463$, in which the combined effects of the TAD and LPS treatment significantly elevated TNF-α gene expression compared to the TAD and saline treatment. Additionally, post-hoc analyses revealed that the TAD and LPS treatment significantly increased TNF-α gene expression compared to the MD and LPS treatment, $p = 0.019$.

A two-way ANOVA also revealed a significant main effect of injection on IL-1β gene expression, $F(1,35) = 113.4$, $p \leq 0.001$, in which mice that received LPS had increased IL-1β gene expression compared to mice that received saline. There was also a significant main effect of diet condition, $F(1,35) = 5.484$, $p = 0.025$, in which mice on the TAD had increased IL-1β gene expression than those on the MD. Finally, the interaction approached significance, $F(1,35) = 4.106$, $p = 0.0504$. Post-hoc analyses revealed that the TAD and LPS treatment significantly increased IL-1β gene expression compared to the MD and LPS treatment, $p = 0.011$.

A two-way ANOVA revealed a significant main effect of injection on IL-6 gene expression, $F(1,37) = 21.22$, $p \leq 0.001$, in which male mice that received LPS had increased IL-6 gene expression compared to male mice that received saline. However, there was no significant main effect of diet on IL-6 gene expression in male mice, $F(1,37) = 0.003$, $p = 0.958$. Additionally, a two-way ANOVA did not reveal a significant main effect of injection, $F(1,22) = 0.001$, $p = 0.98$, or diet condition, $F(1,22) = 3.624$, $p = 0.07$, on IL-10 gene expression in male mice. See Figure 22.

BDNF and BACE1 Expression of mRNA in the Hippocampus of Female Mice.

A two-way ANOVA revealed a significant main effect of injection condition on hippocampal BDNF mRNA in female mice, $F(1, 32) = 4.533$, $p = 0.041$, and this was further supported by a significant interaction, $F(1, 32) = 17.01$, $p \leq 0.001$. However, there was no significant main effect of injection diet condition on BDNF gene expression, $F(1, 32) = 0.000$, $p = 0.9842$, in female mice. Post hoc tests revealed that LPS treatment following long-term TAD consumption decreased gene expression of BDNF in comparison to LPS treatment and long-term MD consumption in female mice, $p = 0.03$. Conversely, the results revealed the opposite effect in saline treated female mice, in which females on the TAD had increased BDNF gene expression compared to those on the MD following saline treatment, $p = 0.032$.

Additionally, a two-way ANOVA revealed a significant main effect of injection on BACE1 gene expression, $F(1,30) = 7.396$, $p \leq 0.01$, in which female mice that received saline treatment had increased BACE1 gene expression compared to female mice that received LPS. Additionally, there was a significant main effect of diet condition, $F(1,30) = 16.860$, $p \leq 0.001$, such that female mice on the TAD had increased BACE1 gene expression compared female mice on the MD. This was further supported by a significant interaction, $F(1,30) = 5.210$, $p = 0.03$. Additionally, post-hoc analyses revealed that the TAD and saline treatment significantly increased BACE1 gene expression compared to MD and saline treatment, $p \leq 0.001$, in female mice. See Figure 23.

Gene Expression of TNF-α, IL-1β, IL-6, and IL-10 in the Hippocampus of Female Mice.

A two-way ANOVA revealed a significant main effect of injection on TNF-α gene expression, $F(1,28) = 49.51$, $p \leq 0.001$, such that female mice that received LPS treatment had increased TNF-α gene expression compared to female mice that received saline treatment. However, there was no significant main effect of diet condition, $F(1,28) = 0.004$, $p = 0.9472$, on TNF-α gene expression in female mice. Additionally, a two-way ANOVA revealed a significant main effect of injection on IL-1β, $F(1,32) = 97.83$, $p \leq 0.001$, such that female mice that received LPS treatment had increased IL-1β gene expression compared to female mice that received saline. However, there was no significant main effect of diet condition, $F(1,32) = 2.247$, $p = 0.144$, on IL-1β gene expression in female mice.

Further, a two-way ANOVA revealed a significant main effect of injection on IL-6 gene expression, $F(1,32) = 82.93$, $p \leq 0.001$, such that female mice that received LPS had increased IL-6 gene expression compared to female mice that received saline. However, there was no significant main effect of diet on IL-6 gene expression in female mice, $F(1,32) = 0.147$, $p = 0.704$. Also, a two-way ANOVA did not reveal a significant main effect of injection, $F(1,22) = 0.057$, $p = 0.814$, or diet condition, $F(1,22) = 0.373$, $p = 0.548$, on IL-10 gene expression in female mice. See Figure 23.

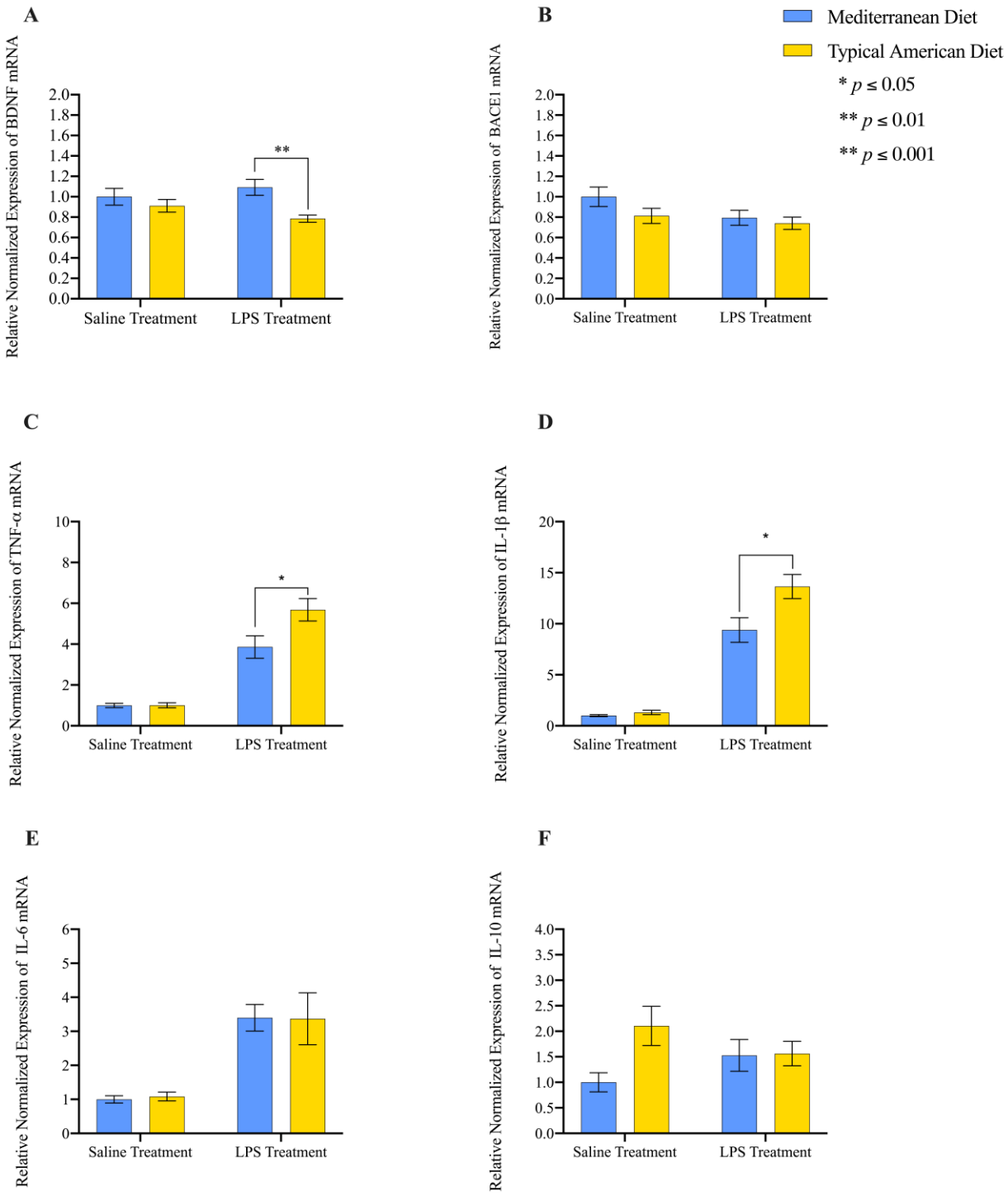


Figure 22. Gene Expression in the Dorsal Hippocampus of Male Mice. (A) Male mice on the TAD had significantly decreased gene expression of BDNF compared to male mice on the MD, $p \leq 0.05$, n's = 9–12. (B) There was no significant main effect of diet on relative normalized gene

expression of BACE1 in male mice, $p > 0.05$, n's = 9–12. (C) Male mice on the TAD had increased TNF-α gene expression compared to male mice on the MD, $p \leq 0.05$, n's = 8–12. (D) Male mice on the TAD had increased IL-1β gene expression compared to male mice on the MD, $p \leq 0.05$, n's = 8–11. (E) There was no significant main effect of diet on relative normalized gene expression of IL-6 in male mice, $p > 0.05$, n's = 8–12. (F) There was no significant main effect of diet on relative normalized gene expression of IL-10 in male mice, $p > 0.05$, n's = 6–8. (A–F) Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

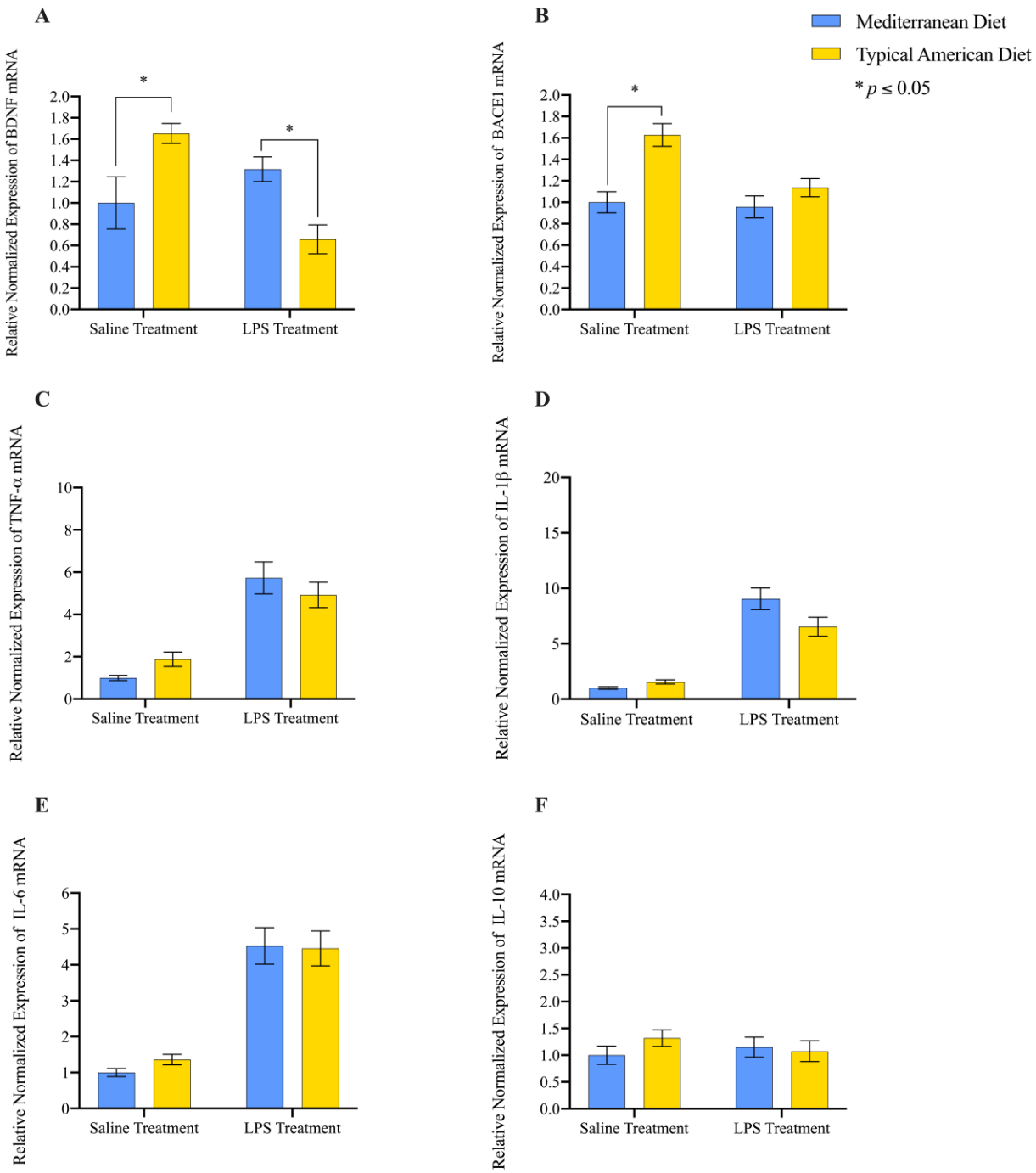


Figure 23. BACE1, BDNF, and Cytokine mRNA Gene Expression in Female Mice. (A) Female mice on the MD had decreased gene expression of BDNF following saline treatment compared to females on the TAD, however, the opposite effect occurred in LPS treated females, *ps* ≤ 0.05, n's = 9. (B) In saline treated females, the MD significantly reduced mRNA expression of BACE1 compared

to the TAD, $p > 0.05$, n's = 8–9. (C) There was no significant main effect of diet on gene expression of TNF-α in female mice, $p > 0.05$, n's = 7–9. (D) There was no significant main effect of diet on gene expression of IL-1β in female mice, $p > 0.05$, n's = 9. (E) There was no significant main effect of diet on gene expression of IL-6 in female mice, $p > 0.05$, n's = 9. (F) There was no significant main effect of diet on gene expression of IL-10 in female mice, $p > 0.05$, n's = 4–8. (A–F) Bars represent mean ± SEM. Significant differences ($p \leq 0.05$) are designated by *.

4. Discussion

The current experiment aimed to understand the potential, neuroprotective effects of the MD against LPS-induced inflammation compared to the TAD, as inflammation is interconnected with AD neuropathology. Prior studies have established that peripheral LPS treatment provokes central inflammation and decreases neurotrophic factors in the hippocampi of C57BL/6 mice (Kahn et al., 2012; Lee et al., 2008). Therefore, we utilized this paradigm to provoke inflammation, and measure the effects of diet and LPS on mRNA gene expression of TNF-α, IL-1β, IL-10, IL-6, BACE1 and BDNF in the dorsal hippocampus.

Previously, studies have shown that long-term consumption of high-fat rodent diets exacerbates mRNA expression of central pro-inflammatory cytokines in wildtype mice (Liu et al., 2014; Pistell et al., 2010), and transgenic mouse models of AD (Walker et al., 2017). However, there is limited data available on the effects of a WD that is more representative of the average American diet on central inflammation in mice (Graham et al., 2016). Graham and colleagues were one of the first research teams to demonstrate that the "average" American diet increased central inflammation compared to a plant-based diet in male C57BL/6 mice and a transgenic mouse model of AD (Graham et al., 2016). Based on the previously discussed findings,

we hypothesized that the TAD would further exacerbate central inflammation following LPS treatment, and the MD would protect the brain following inflammatory insult.

First, our hypothesis that the TAD would increase LPS-induced pro-inflammatory gene expression was partially supported. The results revealed that the MD protected male mice from increased gene expression of TNF-α and IL-1β compared to the TAD. However, our hypothesis that the TAD would increase gene expression of BACE1, and decrease expression of IL-10, was not supported in male mice. Conversely, prior evidence has shown that a WD providing 60% kilocalories from fat increases BACE1 in male C57BL/6 mice (Moser et al., 2018). However, the TAD utilized in the current study only provided 35% kilocalories from fat, and thus, it is difficult to directly compare these results.

We also hypothesized that the TAD would increase mRNA expression of BACE1 in male mice, because it increased soluble $A\beta_{1-42}$. However, these null results suggest that increased soluble $A\beta_{1-42}$ levels in the brain following the TAD may be due to potential, increased transport of peripheral $A\beta_{1-42}$ peptides from other organs, like the liver, rather than increased amyloidogenic cleavage of APP in the brain. BACE1 is largely produced by neurons in the brain, and thus, these data indicate that diet-induced production of $A\beta_{1-42}$ peptides may be occurring in other areas of the body, like the liver (Taylor et al., 2022). BACE1 is also found in adipocytes and hepatocytes, and accordingly, $A\beta_{1-42}$ peptides produced by peripheral cells could potentially cross the BBB via the RAGE receptor and other cellular mechanisms (Deane et al., 2003). In a future study, this hypothesis could be tested by measuring the amount of peripheral $A\beta_{1-42}$ in the blood, white adipose tissue, or liver, to understand the underlying cellular mechanisms of peripheral or central $A\beta_{1-42}$ generation induced by the TAD.

When comparing mRNA expression of pro-inflammatory genes in the hippocampus to serum cytokine levels from Experiment 1, it is interesting to note that the TAD induced a significant amount of serum TNF-α in male and female mice and a moderate, but not significant, amount of serum IL-1β in male mice compared to the MD, whereas we did not see differences in serum IL-10 in male or female mice. Additionally, when measuring IL-6 in male mice, numerous samples fell below the lowest detectable limit of the assay's standard curve, thus preventing analysis or interpretation of these data.

Collectively, Experiments 1 and 2 revealed that the TAD provoked significant TNF-α in serum and the hippocampus, moderate IL-1β in the periphery, and significant IL-1β in the hippocampus in male mice. It is well established that peripheral inflammation can induce central inflammation, either through active transport of cytokines across the BBB, or de novo synthesis of cytokines in the brain via transduction of the vagus nerve (Dantzer & Kelley, 2007; Steinberg et al., 2016; Zanos et al., 2018). Although our hypotheses were not fully supported, a preceding study utilizing a WD with 40% kilocalories from fat reported that a WD did not induce cortical pro-inflammatory cytokines and chemokines compared to a low-fat control diet in male C57BL/6 mice (Pistell et al., 2010). However, they observed significant differences in TNF-α, IL-6, MCP-1, and BDNF in the cortex of mice on an extremely high-fat diet (60% kcal from fat) compared to a low-fat control diet (Pistell et al., 2010), indicating that higher SFA content is more pro-inflammatory in the brain. Interestingly, recent research findings suggest that SFA functions like a TLR4 agonist and increased consumption of SFA activates the immune system similarly to LPS (Huang et al., 2012; Lee et al., 2003). Indeed, treatment with a TLR4 inhibitor has been shown to mitigate inflammation and other harmful effects induced by a high-fat diet in C57BL/6 mice, which provides (Moser et al., 2018).

Moreover, the current study is one of the first studies to illustrate that a comprehensive MD protects male, wildtype mice from LPS-induced gene expression of TNF-α and IL-1β in the hippocampus compared to a macronutrient-matched TAD. Interestingly, male mice on the MD also consumed more food and kilocalories than mice on the TAD but did not gain as much weight. Thus, these data indicate that the *food sources* of macro and micronutrients contribute to neuroinflammation, rather than caloric intake alone.

The current findings are also strengthened by a recent study that compared the effects of corn oil (rich in omega-6) and fish oil (rich in omega-3) supplementation in male C57BL/6 and APP/PS1 mice (Yan et al., 2020). Yan and colleagues (2020) found that corn oil, a common ingredient in the WD, exacerbated NFκB in the hippocampus, and TNF-α and IL-1β in the cortex of APP/PS1 mice compared to fish oil supplementation. Likewise, a prior study also reported that a WD engendered both peripheral and hippocampal inflammation, whereas the supplementation of the plant polyphenol, luteolin (commonly found in fruits and vegetables), attenuated inflammation following WD consumption in male C57BL/6 mice (Liu et al., 2014). Further, supplementation of MD components, like olive oil, has been shown to inhibit LPS-induced inflammation in BALB/c mice (Bitler et al., 2005; Fuccelli et al., 2018) and C57LBL/6J mice (Illesca et al., 2019; Rincón-Cervera et al., 2016).

Moreover, the current results provide support for the previously discussed findings, and also demonstrate that the combined effects of *multiple* MD components, such as MUFAS and diet derived fiber, contributed to the reduction of TNF-α and IL-1β in male mice. Indeed, prior studies have demonstrated that soluble fiber, such as inulin, is anti-inflammatory. The generation of pro-inflammatory cytokines in the gut can induce cytokine production in the brain via transduction of the vagus nerve, and therefore, a healthy gut microbiome could protect the brain from

neuroinflammation (Jena et al., 2018). As fiber is a key aspect of the MD, and has been shown to promote essential, commensal bacteria, such as *Bifidobacterium infantis* (*B. infantis*), it could potentially be utilized in dietary prevention strategies for AD. Notably, a recent study reported that supplementation of *B. infantis* attenuated central TNF-α and IL-6 induced by long-term consumption of the WD in C57BL/6 mice (Jena et al., 2022).

Although we saw differences in mRNA expression of TNF-α and IL-1β in the hippocampus of male mice, we did not see differences in female mice. Likewise, prior studies have reported that the WD induces central inflammation in male C57BL/6 mice, but not in female mice (Graham et al., 2016; Liu et al., 2014; Pistell et al., 2010). Interestingly, a recent study compared the effects of a Western fat blend, with corn oil and soybean oil, to a Mediterranean fat blend, and also found that there was no difference in central TNF-α and IL-1β in a female mouse model of ulcerative colitis (Wenzel et al., 2022). Preceding evidence indicates that there are phenotypic, sexual dimorphisms between male and female C57BL/6 mice following WD consumption, such that a WD does induce behavioral changes, like locomotor activity, in females compared to males (Casimiro et al., 2021). Additionally, prior studies have reported that female C57BL/6 mice do not develop body adiposity (Pereira-Silva et al., 2019), obesity, glucose intolerance, or insulin resistance (Casimiro et al., 2021) following a WD compared to male mice. Based on these findings, we infer that male C57BL/6 mice are more susceptible to negative effects of the WD compared to female C57BL/6 mice, and thus, females of this breed may not be a good model for humans. Thus, potential protective mechanisms in female C57BL/6 mice, such as hormones, should be explored in future research.

Additionally, we hypothesized that the TAD would decrease the expression of BDNF mRNA in the hippocampus compared to the MD. Indeed, in addition to inducing neuroinflammation, Western rodent diets have been shown to reduce proteins related to LTP and

neuronal plasticity, such as acetylcholine, synaptophysin, PSD95, and neurotrophic factors, in the hippocampal and cortical regions of mice (Jena et al., 2022; Jena et al., 2018; Liu et al., 2014; Pistell et al., 2010; Tozuka et al., 2010), and rats (Kanoski et al., 2007; Molteni et al., 2002). Our hypothesis was supported, and there was a significant main effect of diet on BDNF expression in both male and female mice; however, this effect was largely driven by differences found between LPS-treated mice. The results revealed that the MD was protective against LPS treatment in both male and female mice compared to the TAD, and the MD sustained mRNA expression of BDNF after LPS treatment in male mice, specifically. In addition to BDNF's crucial role in synaptic plasticity, it also helps to regulate glucose land metabolic hormones in the brain. The brain utilizes about 20% of the body's total glucose, and thus, it is interesting that male mice on the MD had increased mRNA expression of BDNF in addition to reduced fasting blood glucose levels compared to male mice on the TAD.

Similarly, prior studies have found that supplementation of MD components, like plant polyphenols (Liu et al., 2014) or probiotics (Jena et al., 2022), protects mice from high-fat diet induced BDNF loss in the cortex and hippocampus. These findings are further strengthened by evidence that closer adherence to the MD is associated with increased serum BDNF in adults (Sánchez-Villegas et al., 2011). As AD patients have reduced serum levels of BDNF and increased levels of pro-inflammatory cytokines, (Gezen-Ak et al., 2013), the current results indicate that a MD could perhaps inhibit BDNF loss during an inflammatory state in comparison to a TAD. In the future, we could further assess the therapeutic capacity of the MD against BBB impairment, because damage to the BBB could potentially increase transport of peripheral cytokines and $A\beta_{1–42}$ to the brain (Rutkowsky et al., 2018; Wang et al., 2021), and subsequently reduce BDNF. Specifically, we aim to further explore the underlying cellular mechanisms of

peripheral Aβ transport through RAGE and other receptors on the BBB following MD or TAD consumption, as peripheral activity could be playing a key role in the development of AD neuropathology in the brain.

CHAPTER 4: EXPERIMENT 3, THE EFFECTS OF DIET ON DNA METHLYATION ACROSS MULTIPLE GENERATIONS

1. Introduction

Effects of Perinatal Diet on Behavior and Cognition in Rodent Offspring

Recent evidence suggests that different dietary patterns induce alterations in gene expression and behavior across multiple generations in rodents. It is imperative for researchers to further investigate the effects of maternal or perinatal diet during offspring development, as this is crucial to long-term health later in adulthood. For example, previous experiments have illustrated that a maternal WD induces cognitive dysfunction and behavioral changes in rodent offspring (Cordner et al., 2019; Mischke et al., 2013; Tozuka et al., 2010; Tsan et al., 2021; White et al., 2009). Notably, prior evidence has clearly demonstrated that perinatal exposure to a WD increases anxiety-like behaviors in rodents (reviewed by Tsan et al., 2021). For example, preceding studies have shown that a maternal WD administered to dams throughout gestation and lactation, induces anxiety-like behaviors in rodent offspring that are fed a low-fat, standard diet post weaning (Bilbo & Tsang, 2010; Glendining et al., 2018; Sasaki et al., 2014; Sasaki et al., 2013; Winther et al., 2018).

In addition, researchers have also established that perinatal WD exposure induces cognitive deficits later in life, even after rodents consumed a standard, low-fat diet throughout adulthood (Cordner et al., 2019; Cordner & Tamashiro, 2015; Lépinay et al., 2015; Page et al., 2014; Tozuka et al., 2010). Importantly, the current literature has revealed that perinatal exposure to a WD only impairs particular types of learning and memory, including spatial learning and episodic memory (Boitard et al., 2014; Lépinay et al., 2015; Page et al., 2014; Tozuka et al., 2010).

For example, Tozuka et al. (2010) demonstrated that a Western maternal diet decreased performance during spatial learning tasks in young C57BL/6 offspring. Similarly, other studies have found that exposure to a WD throughout development impaired spatial memory (Boitard et al., 2014; Lépinay et al., 2015; Page et al., 2014) and novel object recognition memory later in adulthood (Cordner et al., 2019; Moreton et al., 2019). Conversely, other studies have demonstrated that maternal WD does not affect cognition during other behavioral tests, such as CFC, in rodent offspring (Peleg-Raibstein et al., 2012; Zieba et al., 2019). Therefore, more research is needed to examine the effects of perinatal WD or TAD exposure on learning and memory processes in different behavioral tests.

Although there is limited research addressing potential transgenerational effects of a complete MD, recent studies have demonstrated that high-fiber maternal diets provide neuroprotection to offspring following birth and throughout adulthood (Liu et al., 2021; Yu et al., 2019). Liu et al. (2021) examined the effects of maternal fiber consumption on behavior and cognition in the offspring of C57BL/6 mice. Researchers administered a standard diet, a high-fat diet, a fiber-supplemented high-fat diet, or a fiber supplemented standard diet to female C57BL/6 mice for three months prior to pairing, and throughout gestation and lactation (Liu et al., 2021). Offspring were weaned and placed on a standard, low-fat diet for five weeks prior to behavioral testing. The results of this study revealed that the maternal high-fiber diet increased novel object discrimination in comparison to the maternal high-fat diet (Liu et al., 2021). Furthermore, the maternal high-fat diet decreased hippocampal BDNF expression, while the maternal high-fat diet, supplemented with fiber, restored BDNF expression in offspring (Liu et al., 2021). These results suggest that fiber plays an important role in learning and memory processes across multiple generations.

Similarly, Yu and colleagues (2019) demonstrated that maternal fiber deficiency induces anxiety-like behaviors, locomotor activity reduction, and cognitive dysfunction in C57BL/6 offspring. However, when offspring were given a butyrate-supplemented diet post-weaning, this reversed the negative effects induced by a low-fiber maternal diet (Yu et al., 2019). Additionally, butyrate supplementation restored spatial memory and increased object exploration time during the novel object recognition test in offspring (Yu et al., 2019). Collectively, these results reveal that exposure to fiber-deficient diets, such as a WD throughout gestation and lactation impairs cognitive and behavioral processes, although butyrate supplementation can provide neuroprotection during development (Yu et al., 2019).

Effects of Perinatal Diet on Inflammation and AD Biomarkers in Rodent Offspring

In addition to inducing anxiety-like behaviors and cognitive deficits, perinatal exposure to a WD has also been shown to increase markers of oxidative stress, inflammation, and activated microglia in rodents (Bilbo & Tsang, 2010; Segovia et al., 2017; White et al., 2009). Bilbo & Tsang (2010) found that a perinatal WD increased expression of microglial cell markers of activation, such as CD11b and TLR4, in the hippocampus, and peripheral inflammatory markers, including IL-1beta and IL-6, later in adulthood. Likewise, White et al. (2009) demonstrated that perinatal exposure to a high-fat diet significantly increased markers of oxidative stress and inflammation, in comparison to perinatal exposure to a low-fat diet, in adult rats.

Prior evidence has also shown that a maternal WD decreases markers of neural plasticity and induces offspring alterations in hippocampal gene expression, therefore suggesting that perinatal exposure to a WD makes the brain more susceptible to AD. Tozuka et al. (2010) found that maternal WD decreased BDNF expression in the hippocampus of young C57BL/6 mice.

Additionally, Cordner et al. (2019) found that a maternal WD induced changes in hippocampal gene expression, such that offspring had decreased expression of insulin, leptin, and glucose transporter 1 receptors in the hippocampus. These data suggest that a WD alters proper regulation and production of insulin, leptin, and glucose, and therefore promotes higher risk of developing diseases such as diabetes mellitus type II and AD (Cordner et al., 2019)

There is limited research on the transgenerational effects of the MD, or MD components, on inflammation and AD pathologies. However, a recent study found that maternal choline supplementation provides protection against AD pathologies in two generations of a transgenic mouse model of AD (Velazquez et al., 2020). Choline is a precursor for acetylcholine production, and as previously discussed, acetylcholine deficiency is interconnected with AD pathologies. Additionally, choline is an important element of the MD, and it is typically found in leafy greens, fish, peanuts, eggs, beans, yogurt, and other dairy products. In this study, dams were administered a choline supplemented diet (5.0g/kg of choline chloride) or a choline control diet (1.0g/kg of choline chloride) for 2.5 months prior to breeding, and throughout gestation and lactation (Velazquez et al., 2020). Following weaning, the first generation (F1) offspring were placed on a standard rodent diet throughout adulthood, and a subset of F1 animals were bred to produce a second generation of animals (F2). The results of this study revealed that maternal choline supplementation attenuated amyloid beta, microglial cell activation, and spatial memory impairment in Morris Water Maze, in both generations of mice (Velazquez et al. 2020). Also, Velazquez et al. (2020) conducted RNA sequencing to examine gene expression in the hippocampal tissue of F1 mice. In addition to providing neuroprotection, maternal choline supplementation changed the expression of 27 genes, many of which were related to immune system processes, in F1 mice (Velazquez et al., 2020). Thus, this study supports the hypothesis

that diet engenders transgenerational changes in mice, and, therefore, that maternal diet could potentially protect offspring from chronic inflammatory diseases, like AD, later in adulthood (Velazquez et al., 2020).

Marchlewicz et al. (2022) studied the effects of a maternal WD versus a maternal MD on body weight, hepatic steatosis, organ weight, and peripheral hormone levels in mouse offspring. The WD was high in SFA and deficient in insoluble fiber, in comparison to the MD that was high in MUFAS and insoluble fiber (Marchlewicz et al., 2022). Dams were placed on one of the two experimental diets for two weeks prior to breeding, and throughout gestation and lactation (Marchlewicz et al., 2022). The results of this study revealed that gestational exposure to the experimental diets did not significantly affect body weight in offspring. Additionally, the MD did not affect body weight, organ weight, or hormone levels (Marchlewicz et al., 2022). However, there were several limitations in this study, for example, the experimental diets did not provide the same amount of energy, and the MD did not provide adequate amounts of soluble fiber. Additionally, dams only consumed the experimental diets two weeks prior to breeding (Marchlewicz et al., 2022). Therefore, future studies need to examine the effects of long-term MD consumption in dams, as short-term feeding may not engender significant changes prior to gestation (Marchlewicz et al., 2022). Additionally, future studies are needed to explore the effects of typical American and Mediterranean diets that more closely resemble real human dietary patterns in animals (vs. exaggerated versions), as diet could be utilized as a potential strategy to prevent or slow down the progression of AD.

Epigenetic Effects of Diet on Inflammation and AD Biomarkers

Epigenetics is the study of phenotypic changes that involve altered gene expression by modifications to the genome without affecting the gene's sequence, yet most often lead to

alterations in gene expression. Heritable changes in cellular and physiological phenotypic traits can also be categorized as epigenetic and may be induced as a result of environmental factors. Several different changes to the genome can affect gene expression and function, including DNA methylation and histone modifications. For example, prior research has demonstrated that DNA methylation occurs in genes related to inflammation and cognition, suggesting that epigenetics may play a role in the development of neurodegenerative diseases, such as AD (Conole et al., 2021). Specifically, DNA methyltransferases (DNMT) are proteins that play a key role in DNA methylation (Athanasopoulos et al., 2016), and prior research in human subjects has shown that a polymorphisms in the gene DNMT3B leads to altered DNA methylation activity and is associated with AD (Pezzi et al., 2014). These types of changes can last through multiple cell divisions and throughout the life of the cell. Further, these changes can be passed on to multiple generations, often termed transgenerational epigenetic inheritance. Hence, future research could further explore potential AD interventions strategies that target the epigenome, as this could possibly provide AD protection to multiple generations.

Several environmental factors, such as diet, may contribute to epigenetic modifications in genes associated with inflammation and AD. For example, several Mediterranean dietary factors, such as omega-3 fatty acids, resveratrol, and oleuropein (a plant polyphenol found in olive oil), have been shown to induce DNA methylation or histone modifications (reviewed in Athanasopoulos et al., 2016). As previously discussed, prior research has demonstrated that perinatal WD exposure induces changes in gene expression, specifically in genes related to oxidative stress, inflammation, and microglial cell activation (Bilbo & Tsang, 2010; Segovia et al., 2017; White et al., 2009). Additionally, other researchers have discovered that perinatal WD exposure alters BDNF gene expression (Tozuka et al., 2010), and the expression of genes related to insulin, leptin, and glucose regulation (Cordner et al., 2019). Thus, this previous work suggests that a perinatal engenders epigenetic modifications, although this hypothesis is not

confirmed, because epigenetic alterations were not measured in these studies (Bilbo & Tsang, 2010; Cordner et al., 2019; Segovia et al., 2017; Tozuka et al., 2010; White et al., 2009).

As previously discussed, Velazquez et al. (2020) explored the effects of choline supplementation across multiple generations of a transgenic mouse model of AD. The F1 offspring were only exposed to perinatal choline supplementation, and the F2 offspring were never exposed to choline supplementation (Velazquez et al., 2020). Notably, the F1 offspring displayed changes in inflammatory gene expression, and gene expression was not measured in the F2 offspring (Velazquez et al., 2020). However, the F2 offspring did have a reduction of amyloid beta plaques in the hippocampal region, suggesting that choline supplementation induced epigenetic effects in F1 offspring, which was thus inherited by F2 offspring. However, this hypothesis was not confirmed by DNA methylation analysis. Therefore, future research is needed to examine the heritability of epigenetic effects of choline supplementation across multiple generations, to confirm that maternal choline supplementation induces epigenetic changes in germ cells, prior to pregnancy, that is then inherited by offspring.

Furthermore, other studies have found that dietary compounds added to *in vitro* cultures (Flores-Sierra et al., 2016; Zhao et al., 2018) and dietary changes *in vivo* (Dudley et al., 2011; Gali Ramamoorthy et al., 2018) induce epigenetic effects via DNA methylation or histone acetylation. For example, Zhao and colleagues (2018) examined the epigenetic effects of sulforaphane, a compound found in cruciferous vegetables that is abundantly present in the MD. The results of this study revealed that *in vitro* sulforaphane treatment increased Nrf2 expression by attenuating DNA methylation in the Nrf2 gene's promoter region. Nrf2 is a transcription factor that is responsible for activating important antioxidant responses and protecting the cell from oxidative stress (Zhao et al., 2018). Therefore, sulforaphane treatment could be used as a potential treatment for oxidative stress. Additionally, other *in vitro* studies have demonstrated that oleic acid and resveratrol, two key plant polyphenols in the MD, induce epigenetic changes

via DNA methylation and histone protein modifications (Griñán-Ferré et al., 2021; Silva-Martínez et al., 2016; Stefanska et al., 2010). Further evidence has also illustrated that low adherence to the MD throughout pregnancy induces DNA methylation in mother's infants (Gonzalez-Nahm et al., 2017). Collectively, the previously discussed studies demonstrate that several components of the MD induce epigenetic changes.

Finally, *in vivo* studies have also revealed that perinatal exposure to high-fat diets induce epigenetic changes via DNA methylation in rodents (Dudley et al., 2011; Gali Ramamoorthy et al., 2018). For example, Gali-Ramamoorthy and colleagues (2018) demonstrated that a maternal high-fat diet induced hypermethylation in the enhancer and promoter regions of the hypothalamic *POMc* gene, which is related to energy balance and leptin regulation. Therefore, perinatal high-fat diet exposure may alter genes related to energy, hunger, and satiety signals (Gali-Ramamoorthy et al., 2018). Overall, nutrition is a key contributor to epigenetic changes that could contribute to disease pathology in old age, although there is, once again, limited research on the epigenetic effects of the *whole* MD, and more research is needed to contribute to this literature.

Hypotheses

We hypothesized that both diets (MD or TAD) would induce epigenetic changes via DNA methylation in the parent generation (G1) and their offspring (F1). We hypothesized that the TAD would increase the expression of genes related to inflammation and oxidative stress, whereas the MD would induce DNA methylation in the promoter regions of these genes to silence them, or potentially upregulate DNA methylation to counteract the effects of genes.

2. Materials and Methodology for Experiment 3

A subset of male and female mice from study one was paired between three and four months of age for breeding purposes. Females on the MD were paired with males on the MD, and females on the TAD were paired with males on the TAD. All breeders continued to consume their assigned experimental diets throughout breeding, gestation, and lactation, until nine months of age. Following perinatal diet exposure, all offspring (F1) were weaned onto a standard, low-fat rodent diet (LFD) (Prolab RMH 1800-5LL2; LabDiet, St. Louis, MO), at postnatal day 21. See table 3 for LFD formula and key ingredients and Figure 24 for experimental timeline.

LFD Formula		
	kcal%	**Key Ingredients**
Protein	21.114	Fish meal, dried whey, porcine meat and bone meal, L-lysine
Carbs	65.128	Ground corn, wheat middling, soybean meal
Fat (Total)	13.758	Porcine animal fat, soybean oil
SFA	3.3	
MUFA	3.99	
PUFA	2.89	
Ratio *n*-6/*n*-3	7.22:1	
Insoluble Fiber	12.1	
Soluble Fiber	1.53	

Table 3. Standard, Low-Fat Rodent Diet Formula and Key Ingredients.

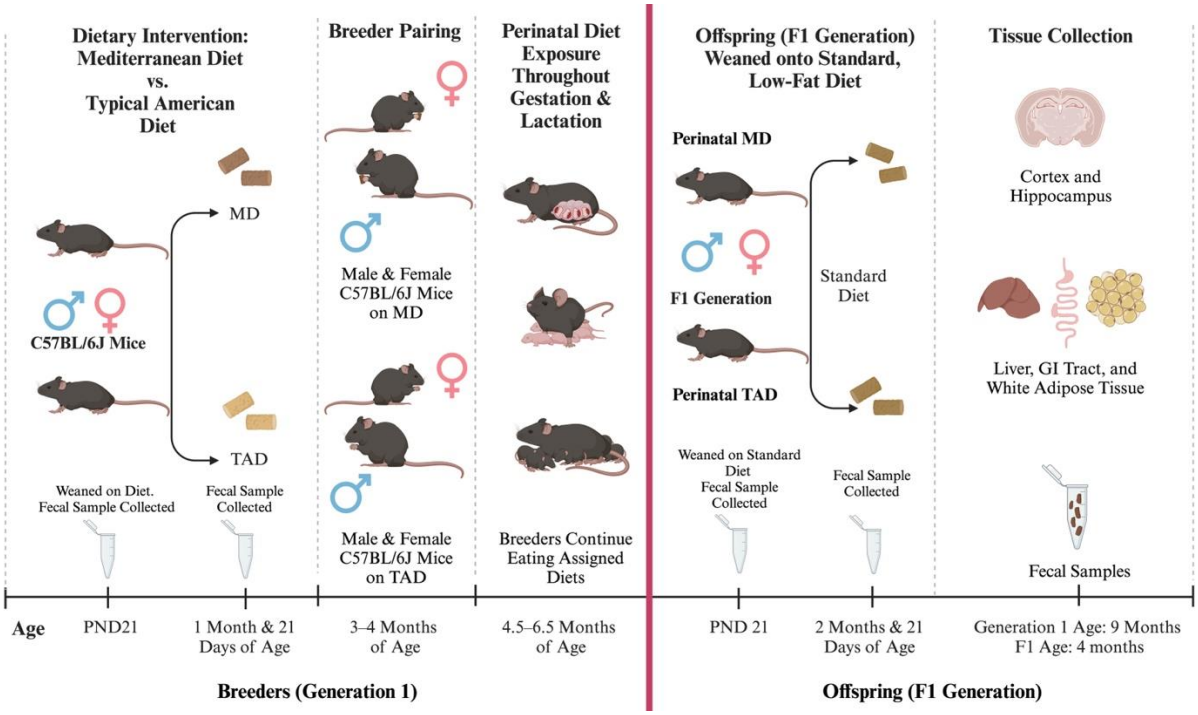


Figure 24. Experimental Timeline for Experiment 3.

Fecal Sample Collections

F1 fecal samples were collected at two different timepoints: at weaning (PND21), and two months and 21 days of age. Subsequently, samples were stored at -80 degrees Celsius. Microbiome analyses were conducted by our collaborators in the Allen Lab (University of North Texas, Health Science Center).

Tissue and Serum Collection

Tissue and serum were collected from both the parental generation (G1) and their offspring (F1) following approximately eight months of MD or TAD administration, or three months of LFD administration, respectively. First, the hippocampus was collected and genomic DNA was isolated from the hippocampus using the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD,) and stored at –80° C for later analysis. Second, the cortex was collected and

placed in RNA later for future gene expression analysis. Experimenters also collected serum, liver, spleen, and white adipose tissue.

DNA Methylation

Complete DNA sample sets were transported to the University of Texas Southwestern Medical Center Microarray Core Facility (Dallas, TX). Experimenters utilized the Infinium Mouse Methylation Beadchip (Illumina, San Diego, California), and measured DNA methylation at over 285,000 different loci sites across the mouse genome. We examined two different types of methylation patterns within the dataset: (1) differentially methylated loci (DML), indicating that DNA methylation occurred in one, individual locus (one base pair at the exact location of the gene of interest on the chromosome), and (2) differentially methylated regions (DMR), indicating that methylation occurred at multiple locus sites within the genome. This latter method has been utilized to measure epigenetic changes in the post-mortem brains of AD patients, as the discovery of DMLs and DMRs associated with AD can help researchers better understand the neuropathology of AD (Li et al., 2020).

Statistical Analysis

The mouse methylome data was analyzed by Dr. Matthew Hale (TCU, Biology) and his graduate student, Bridey Brown, in R version 4.3.1 with the R-package Sensible Step-wise Analysis of DNA Methylation BeadChips (*SeSAMe*) version 1.18.4. Default data preprocessing codes were used to perform non-linear dye-bias correction, p-value detection, and noob background correction. The Benjamini–Hochberg correction was utilized to decrease potential, false discovery rates.

Results

The current experiment examined the effects of life-long diet (MD or TAD) in the parental generation (G1 male and female mice), and perinatal diet exposure (MD or TAD) in the offspring (F1 male and female mice) on DNA methylation across the mouse epigenome. The DNA samples have already been processed, and the mouse epigenome is currently being investigated. Due to the large size of this data set, we are still in the initial stages of statistical analysis. However, we have discovered some trends in the parental generation data so far, and these results are reported in Table 4.

Gene	Gene Description	Methylation Pattern	Results	References
Adgrb2	• Thought to regulate g-protein coupled receptor activity and synaptogenesis	DMR	Hypomethylation in G1 Mice on MD	*(Adgrb2 adhesion G protein-coupled receptor B2 [Mus musculus (house mouse)], 2023)*
Azi2	• Plays a role in NFκB signaling, cytokine production (TNF-α, type II interferon, IL-6), T cell activation, and dendritic cell generation.	DML	Hypermethylation in G1 Mice on MD	*(Azi2 5-azacytidine induced gene 2 [Mus musculus (house mouse)], 2023)*
Camk2g	• Helps to regulate neuron projection development and cardiac muscle relaxation.	DMR	Hypomethylation in G1 Mice on MD	*(Camk2g calcium/calmodulin-dependent protein kinase II gamma [Mus musculus (house mouse)], 2023)*

Celf2 (also known as CUGBP2)	• Plays a role in RNA binding and mRNA splicing.	DMR	Hypomethylation in G1 Mice on MD	(*Celf2 CUGBP, Elav-like family member 2 [Mus musculus (house mouse)]*, 2023)
E2f5	• Transcription factor • Promotes DNA-binding. • Contributes to normal development during morphogenesis. • Largely expressed in the choroid plexus and utilized to study hydrocephalus. • Contributes to the development of the male reproductive tract.	DMR	Hypomethylation in G1 Mice on MD	(*E2F transcription factor 5 [Mus musculus (house mouse)]*, 2023)
Fam83b	• Thought to potentially play a role in cell proliferation. • Potentially thought to provoke binding of epidermal growth factor.	DMR	Hypomethylation in G1 Mice on MD	(*family with sequence similarity 83, member B [Mus musculus (house mouse)]*, 2023)
Foxa1	• Facilitates chromatin binding and remodeling. • It has been shown to regulate glucocorticoid via chromatin opening	DMR	Hypomethylation in G1 Mice on MD	(*forkhead box A1 [Mus musculus (house mouse)]*, 2023) (Belikov et al., 2009)
Grin3a	• Facilitates NMDA receptor activity • Involved in calcium channel and transmitter-gated ion channel activities • Facilitates postsynaptic membrane potential and synaptic vesicle exocytosis	DMR	Hypomethylation in G1 Mice on MD	(*Grin3a glutamate receptor ionotropic, NMDA3A [Mus musculus (house mouse)]*, 2023)

Lrp1b	• Lrp1b is in the LDL receptor family • Primarily expression in the brain in mice • Involved in cholesterol and lipid transport • Regulates efflux transport of Aβ from the brain	DML	Hypermethylation in G1 Mice on MD	(*Lrp1b low density lipoprotein-related protein 1B [Mus musculus (house mouse)]*, 2023) (Li et al., 2005)
Lrrc49	• Found in the cytoplasm and thought to facilitate in dynein arm structure in the cytoplasm	DML	Hypermethylation in G1 Mice on MD	(*Lrrc49 leucine rich repeat containing 49 [Mus musculus (house mouse)]*, 2023)
Nob1	• Thought to regulate endoribonuclease activity • Expressed in the retina and involved in visual perception	DMR	Hypomethylation in G1 Mice on MD	(*NIN1/RPN12 binding protein 1 homolog [Mus musculus (house mouse)]*, 2023)
Stard3	• Thought to engender cholesterol binding • Expressed in the CNS and PNS • Has been shown to help combat oxidative stress and inflammation induced by LDL *in vitro*	DML	Hypermethylation in G1 Mice on MD	(*Stard3 StAR related lipid transfer domain containing 3 [Mus musculus (house mouse)]*, 2023) (Almarhoun et al., 2021)
Txnrd3	• Facilitates oxidoreductase activity • Involved in several processes including redox homeostasis, cell differentiation and sperm development	DML	Hypermethylation in G1 Mice on MD	(*Txnrd3 thioredoxin reductase 3 [Mus musculus (house mouse)]*, 2023)

Zfp454	• Potentially involved in transcription regulation (RNA polymerase specific)	DMR	Hypomethylation in G1 Mice on MD	(*Zfp454 zinc finger protein 454 [Mus musculus (house mouse)]*, 2023)

Table 4. DNA methylation trends in male and female mice from the parental generation.

Following life-long diet consumption, from PND21 to approximately 9 months of age, G1 mice were euthanized, and DNA was immediately isolated from the hippocampus of each mouse. Experimenters utilized the Infinium Mouse Methylation Beadchip (Illumina, San Diego, California) and measured DNA methylation at over 285,000 different locus sites across the mouse genome. Note that hypermethylation in the promoter region likely leads to decreased transcription of the gene, whereas hypomethylation in the promoter region likely leads to increased gene transcription. Differential gene expression will be measured and confirmed in future experiments via qRT-PCR. *Abbreviations: DML = differentially methylated locus, DMR = differentially methylated region.

Discussion

The current trends in the dataset revealed that several genes were significantly hypomethylated or hypermethylated following life-long consumption of the MD, compared to the TAD, in both male and female C57BL/6J mice in the parent generation (G1). First, we discovered that several genes involved in the inflammatory response were hypermethylated in mice on the MD, including Azi2, Stard3 and Celf2, indicating that there was potential decreased transcription of these genes in the promoter region. Interestingly, Azi2 plays a role in in NFκB signaling and pro-inflammatory cytokine production, like TNF-α (*Azi2 5-azacytidine induced gene 2 [Mus musculus (house mouse)]*, 2023). Based on our findings in Experiments 2 and 3, we hypothesize that mRNA expression of TNF-α in the hippocampus was potentially decreased

due to hypermethylation of Azi2, although this hypothesis will need to be confirmed via qRT-PCR. Additionally, prior evidence suggests that Celf2 (also known as CUGBP2) plays a role in the anti-inflammatory response. For example, the impairment of Celf2 (via CELF2-pLX-sgRNA viruses) has been shown to hinder IL-10's ability to reduce TNF-α expression in raw 246.7 macrophages (Yoon et al., 2020). The current data revealed that the MD induced methylation around the Celf2 gene, however, we do not know if this enhancing or suppressing Celf2 gene transcription, and thus, we will need to confirm this via qRT-PCR.

Also related to our previous findings, the current data revealed that there was increased hypermethylation of LRP-1b, an LDL receptor involved in cholesterol and lipid transport, in mice on the MD. LRP is a receptor that is commonly found on endothelial cells that help make up the BBB. LRP-1b is a part of the LRP family, and it is one of several members that share similar functions. For example, LRP family members have been shown to regulate the efflux of Aβ away from the brain (Li et al., 2005). Based on our previous findings in experiment one, in which we found that the TAD increased soluble $A\beta_{1–42}$, it makes sense that we are seeing significant differences in methylation patterns of LRP-1b between mice on the MD and TAD. In the future, we will measure gene expression of LRP-1b via qRT-PCR to understand if hypermethylation increased or decreased LRP-1 gene expression following the MD.

Interestingly, two Mediterranean dietary factors have been shown to increase LRP-1 in C57BL/6 mice and transgenic mouse models of AD, including components in olive oil (Abuznait et al., 2013) and fish oil (Yan et al., 2020). For example, Abuznait et al. (2013) demonstrated that a plant polyphenol found in olive oil, oleocanthal, increased LRP1 expression in cultured, mouse endothelial cells *in vitro*, and increased $A\beta_{1–40}$ clearance in the brains of C57BL/6 mice (Abuznait et al., 2013). Based on these previous findings, we hypothesize that differential

hypermethylation of LRP-1b occurred in the coding region, thus increasing LRP-1b gene expression and clearance of potential $A\beta_{1-42}$ in the hippocampi of mice on the MD, compared to the TAD. Further, we hypothesize that LRP-1b expression was potentially decreased in mice on the TAD because there were higher levels of soluble $A\beta_{1-42}$ in the cortex and hippocampus of male mice on the TAD.

We also discovered that the MD induced significant differential methylation patterns in genes that play a role in synaptogenesis (*Adgrb2 adhesion G protein-coupled receptor B2 [Mus musculus (house mouse)]*, 2023), neuron projection development (*Camk2g calcium/calmodulin-dependent protein kinase II gamma [Mus musculus (house mouse)]*, 2023), cell proliferation (*family with sequence similarity 83, member B [Mus musculus (house mouse)]*, 2023), morphogenesis and CSF regulation in the choroid plexus (*E2F transcription factor 5 [Mus musculus (house mouse)]*, 2023; Lindeman et al., 1998), NMDA receptor activity, calcium channel and transmitter-gated ion channel activities, postsynaptic membrane potential, and synaptic vesicle exocytosis (*Grin3a glutamate receptor ionotropic, NMDA3A [Mus musculus (house mouse)]*, 2023). Based on the functions of these genes, it makes sense that we have discovered trends in DNA methylation in genes involved in the neural mechanisms of learning and memory, such as NDMA activity facilitated by Grin3a. However, these trends will require further analysis and confirmation via qRT-PCR to understand how methylation patterns affected gene expression.

CHAPTER 5: GENERAL CONCLUSION

AD prevalence is projected to increase by over 130% in the U.S. by the year 2060 ("2023 Alzheimer's disease facts and figures," 2023). In addition to the physiological and psychological burden that AD patients and their family members experience, this substantial rise in AD cases has also been exceptionally monetarily expensive. In 2021 alone, the cost of healthcare for AD patients exceeded 355 billion dollars ("2021 Alzheimer's disease facts and figures," 2021). Indeed, researchers estimate that annual AD healthcare will cost Americans over one trillion dollars by the year 2050 ("2021 Alzheimer's disease facts and figures," 2021).

Right now, *preventative* healthcare is absolutely urgent and crucial. As previously stated, the Lancet commission reported that approximately 40% of all dementia cases could potentially be delayed or prevented by reducing modifiable risk factors, like mid-life obesity (Livingston et al., 2020). Therefore, it is unquestionably pressing for researchers to investigate preventative strategies, like the implementation of healthy dietary patterns, to target risk factors and hinder the predicted, consequential rise of AD cases in the U.S. The Western dietary pattern, consumed by most Americans, has been associated with an increased risk of obesity and AD development (Baranowski, Hayward, et al., 2018; Morris & Tangney, 2014; Whitmer et al., 2007), whereas, the MD has been associated with decreased obesity (Poulimeneas et al., 2020; Sánchez-Villegas et al., 2006; Schröder et al., 2004), AD risk (Gu et al., 2010), and global AD pathologies (Agarwal et al., 2023). Hence, the current experiments aimed to contribute to this research cause by investigating the potential, protective effects of a comprehensive MD compared to a macronutrient-matched TAD on AD biomarkers in C57BL/6 mice.

In Experiments 1 through 3, we aimed to address several limitations in the current scientific literature. First, we designed one of the first comprehensive Mediterranean rodent diets

that mimicked the typical MD, to study AD susceptibility in wildtype mice. Rather than examine one or two key components of the MD, like fatty acids or fiber, we wanted to explore how the *synergy* of numerous MD factors play a role in disease prevention. Second, we created a rodent diet that was more representative of the TAD, rather than a WD with extremely high, exaggerated fat or sugar content. Similarly, Graham and colleagues (2016) were the first to design a Western rodent diet that was more representative of the average American diet, and Shively and colleagues were the first research team to create two, macronutrient-matched Mediterranean and Western diets for non-human primates (C. A. Shively et al., 2020; Shively et al., 2019; Carol A. Shively et al., 2020). Like Shively and colleagues, we also designed two macronutrient-matched diets to control for caloric availability, unlike most studies that have typically compared high-fat WDs to low-fat control diets in rodents.

Collectively, the results of Experiment 1 revealed that the MD protected male mice from (1) significant weight gain, (2) excess fat deposition in the abdomen and liver, (3) serum TNF-α, (4) soluble $A\beta_{1-42}$ in the cortex and hippocampus, (5) anxiety-like behavior, and (6) spatial memory impairment compared to the TAD. Additionally, the MD protected female mice from (1) excess fat deposition in the liver, (2) serum TNF-α, (3) and soluble $A\beta_{1-42}$ in the cortex compared to the TAD. In contrast to the differences observed in male mice, there were no differences in food consumption, body weight, organ weight, hippocampal $A\beta_{1-42}$, exploratory and anxiety-like behavior, or spatial memory between females on the MD and TAD. Moreover, male mice on the MD consumed more kilocalories, despite having a lower body weight, and exhibited increased exploratory and locomotor activity compared to male mice on the TAD.

Importantly, the results of the current study fill an important gap in the current literature and indicate that a comprehensive MD prevents soluble $A\beta_{1-42}$ in both the cortex and

hippocampus, and spatial memory impairment in the OLM task, compared to a macronutrient-matched TAD in male mice. Taken together, these data suggest that the implementation of the MD early in life, along with long-term adherence to the MD throughout adulthood, prevents AD biomarkers and spatial memory impairment, compared to the TAD in male C57BL/6 mice. As we did not utilize a transgenic mouse model of AD in the current set of experiments, this suggests that long-term consumption of a TAD could potentially increase risk of AD and cognitive dysfunction without genetic predisposition. Over 94% of all AD cases are sporadic (SAD), and, thus, these results provide more support for the hypothesis that Western-style diets like the average American diet, could potentially be a causal trigger in the development of SAD later in life.

Further, the results of Experiment 2 demonstrated that long-term consumption of the MD protected male mice from LPS-induced gene expression of TNF-α and IL-1β in the hippocampus compared to the TAD. However, there were no differences in BACE1 or IL-10 gene expression in male mice. Additionally, the results revealed that the MD sustained BDNF levels in the hippocampus of male mice following LPS treatment, compared to the TAD. These findings contribute to previous research that also demonstrated the harmful effects of LPS treatment or WD consumption on BDNF in rodents. Interestingly, within saline-treated females, the TAD increased BDNF expression compared to the MD, whereas within LPS-treated females, the MD increased BDNF expression compared to the TAD. These data indicate that the MD provides protective benefits against BDNF loss following an inflammatory trigger. Further, although we did not see differences in BACE1 gene expression in males, we found that females on the MD had reduced BACE1 gene expression compared to females on the TAD.

For Experiment 3, sample processing and data analysis are still in progress. We sent fecal samples collected from G1 and F1 mice to our collaborators in the Allen Lab (University of North Texas, Health Science Center), to measure microbial diversity across multiple generations, and we aim to analyze and publish these data by next year. Additionally, we measured DNA methylation across multiple generations utilizing a microarray analysis that examines methylation at over 285,000 different loci across the mouse genome. We are currently analyzing these data and plan to report our findings next year.

Limitations and Future Directions

There were limitations in the current experiments that should be addressed in future research. First, we aimed to design two novel, experimental rodent diets that mimicked human diets in pellet form. We could not include every aspect of the MD or TAD in pellet form. Obviously whole fruits, vegetables, seafood, red wine, and other key components of the MD were not included in our diet formulation, although we tried to represent them as best as possible. For example, although we did not provide the mice with whole fruits and vegetables, we provided them with complex carbohydrates from brown rice, and dietary fiber from inulin, psyllium, and cellulose to mimic nutrients that would be attained from fruits and vegetables.

In the future, it would be important to incorporate fruits and vegetables in a comprehensive Mediterranean diet, similar to a formulation created by Shively and colleagues for non-human primates (Frye et al., 2021; Johnson et al., 2022; Johnson et al., 2021; Latimer et al., 2019; C. A. Shively et al., 2020; Shively et al., 2019; Carol A. Shively et al., 2020). Interestingly, Shivley, Frye, and Johnston designed two, macronutrient-matched MD and WDs for non-human primates, and their experimental diet formulations were very similar to those used in the current study. However, in the MD formula, they added walnut powder, black and garbanzo beans, and banana

and apple sauce, and these ingredients would have strengthened the MD formula utilized in the current study. In the future, we could potentially add plant polyphenols to the MD to better mimic the human MD. For example, Piazzi and colleagues (2019) created a phytochemical blend from numerous sources, such as apples and walnuts, to mimic the diverse polyphenol intake of people in Mediterranean regions. However, the experimenters supplemented a WD or low-fat control diet with the plant polyphenol blend and did not aim to examine the effects of phytochemicals in a comprehensive Mediterranean rodent diet. It would be interesting to investigate the effects of the comprehensive Mediterranean rodent diet utilized in the current study, supplemented with a plant polyphenol blend created by Piazzi's research team (Piazzi et al., 2019).

Another limitation of the current study is that we utilized female C57BL/6 mice to study the effects of the WD, and prior research has shown that female C57BL/6 mice are protected from WD-induced obesity, insulin resistance, and behavioral changes compared to males (Casimiro et al., 2021). It is important to note that female C57BL/6 mice do not experience estrogen loss at mid-life like humans, and thus, this mouse strain might not be the best representative model for females (Nelson et al., 1981). Estrogen is crucial for learning and memory processes, and it is well established that estrogen increases neurogenesis, LTP (Córdoba Montoya & Carrer, 1997; Daniel et al., 1997; Foy et al., 1999), and dendritic spine density (Gould et al., 1990; Woolley et al., 1990) in female rodents.

Accordingly, a future direction of the current study is to examine the effects of the MD versus the TAD in female C57BL/6 mice following chemically induced menopause via 4-vinylcyclohexene diepoxide (VCD). Our laboratory will investigate the effects of the chemical, VCD, which is a commonly used and verified model for menopause in rodents (Brooks et al.,

2016). We hypothesize that estrogen played a neuroprotective in female C57BL/6 mice in the current study, and thus, we infer that combined treatment of VCD and the WD will promote Aβ and neuroinflammation compared to the MD. Prior studies in female APOE4 knock-in mice demonstrated that combined WD and VCD treatment increased cognitive deficits, and reduced BDNF and DHA in the brain, whereas fish oil supplementation protected mice from the combined effects of WD and estrogen loss via VCD (Pontifex et al., 2022; Pontifex et al., 2021). Interestingly, the proposed, future study will allow our laboratory to further explore phenotypic and biological sexual dimorphisms in C57BL/6 mice following dietary manipulations (Casimiro et al., 2021). As AD largely affects more women than men, our laboratory posits that exploring the combined effects of diet and menopause on AD neuropathology is a crucial next step for this research project.

Experiments 1 through 3 collectively revealed that the TAD induced AD risk factors, including elevated body weight and fatty liver, in male mice. Increased adiposity was strongly correlated with elevated serum TNF-α and cortical $A\beta_{1–42}$, suggesting that the TAD provoked AD biomarkers compared to the MD. Our hypothesis posits that liver damage may impair peripheral $A\beta_{1–42}$ clearance, potentially enhancing $A\beta_{1–42}$ transport across the BBB through receptors like RAGE. This process may contribute to peripheral and central inflammation. Supporting this hypothesis, long-term MD consumption induced DNA methylation of the LRP-1b gene, which is pivotal in $A\beta_{1–42}$ clearance in the brain. The observed relationship between the gut microbiome, liver, and brain in a pathogenic state (Figure 25) underscores our hypothesis that the MD shields against AD neuropathology by preventing adiposity and fatty liver. Simultaneously, we hypothesize that the MD increased LRP-1b gene expression and facilitated $A\beta_{1–42}$ clearance. Our future experiments aim to delve deeper into these relationships and their

potential contributions to AD neuropathology. These findings carry significance, as they highlight modifiable risk factors that are correlated with increased AD-related markers in a sporadic AD (SAD) model. Given that SAD represents over 94% of total AD cases, our results strengthen existing evidence linking adiposity and fatty liver with SAD susceptibility, and provide more insight on the protective capacity of the MD against AD risk factors.

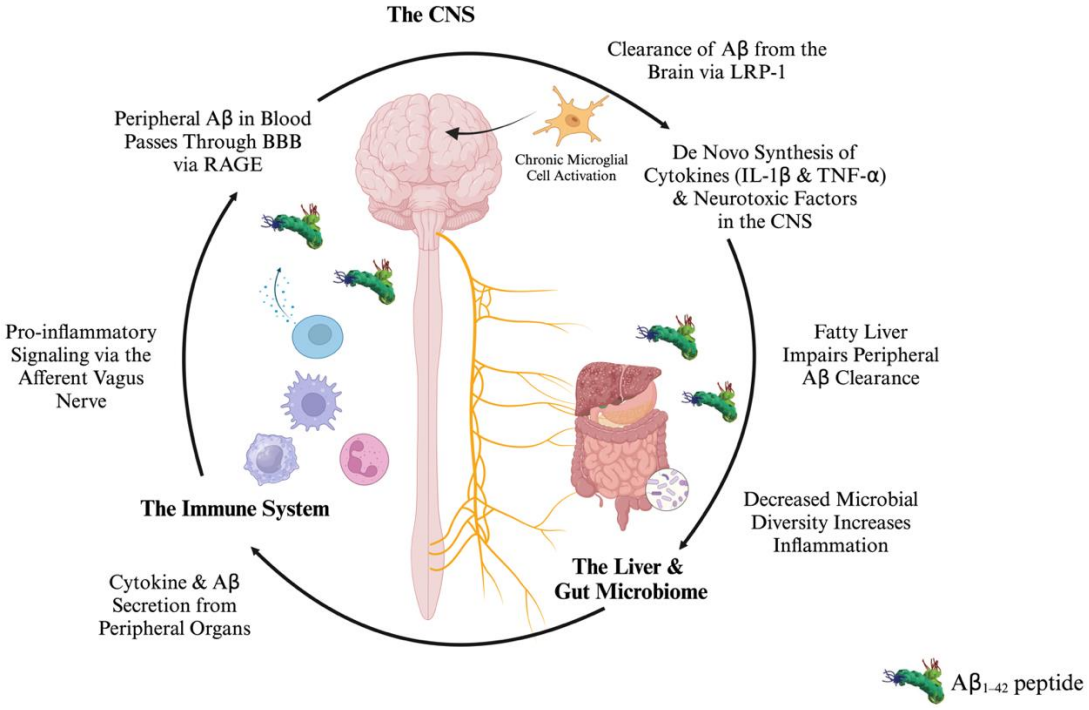


Figure 25. The Liver, Gut and Brain Axis Contributes to AD Neuropathology. Created with BioRender.

Appendix

Ingredients (grams)	Mediterranean Diet	Typical American Diet
Protein Sources		
Casein	0	165.5
Soy Protein	47	0
Fish Protein	47	0
Egg White	47	0
DL-methionine	1.9	0.3
L-Cystine	3	3
Carbohydrate Sources		
Corn Starch	0	370.5
Brown Rice Flour	205	0
Wheat Starch	205	0
Maltodextrin 10	125	125
Dextrose	0	0
Fiber Sources		
Cellulose, BW200 (insoluble)	50	50
Psyllium (soluble)	50	0
Inulin (soluble)	50	0
Fat Sources		
Soybean Oil	0	0
Corn Oil	0	0
Safflower Oil	0	12
Beef Fat, Bunge	3	106.3
Butter, Anhydrous	16	34
Flaxseed Oil	7	0
Menhaden Oil, ARBP-F	10	0
Olive Oil	107	0
Other Micronutrients		
Mineral Mix S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Biotin (1%)	0.1	0
Choline Chloride	3	1.07
Total	**1032.0000 gms**	**922.672 gms**

Table 5. Experimental Diet Ingredients.

Macronutrients	Mediterranean Diet	Typical American Diet
Macronutrients (grams)		
Protein (gms)	147.2	147.3
Carbohydrate (gms)	495.3	495.5
Fat (gms)	153.2	153.1
Total Fiber (gms)	153.7	50.0
Insoluble Fiber (gms)	85.4	50.0
Soluble Fiber (gms)	59.4	0.0
Macronutrients g%		
Protein	14.3	16.0
Carbohydrate	48.0	53.7
Fat	14.8	16.6
Total Fiber	14.9	5.4
Insoluble Fiber	8.3	5.4
Soluble Fiber	5.8	0.0
Macronutrients kcal		
Protein	*589*	*589*
Carbohydrate	*1981*	*1982*
Fat	*1379*	*1378*
Total kcal	***3949***	***3949***
Macronutrients kcal%		
Protein	*15*	*15*
Carbohydrate	*50*	*50*
Fat	*35*	*35*
Fats (grams)		
Saturated Fatty Acids	29.5	74.4
Monounsaturated Fatty Acids	86.2	54.0
Polyunsaturated Fatty Acids	24.4	14.1
n-6	15.9	13.4
n-3	8.0	0.9
n-6/n-3 Ratio	2.0	15.6
Fats kcal%		
Saturated Fatty Acids	6.7	17.0
Monounsaturated Fatty Acids	19.6	12.3
Polyunsaturated Fatty Acids	5.6	3.2

Table 6. Experimental Diet Macronutrients.

Micronutrients	Mediterranean Diet	Typical American Diet
Other ingredients		
tBHQ, mg	2.00	2.00
Added Choline, g	2.25	0.80
Amino Acids, g/kg		
Histidine	3.0	4.1
Isoleucine	6.4	6.6
Leucine	10.1	14.0
Lysine	8.7	11.7
Methionine	4.8	4.8
Phenylalanine	6.0	7.5
Threonine	5.6	6.5
Tryptophan	1.5	1.8
Valine	6.6	8.3
Cystine	4.8	4.3
Tyrosine	4.5	8.1

Table 7. Experimental Diet Micronutrients.

Ingredients Providing Fat (grams)	**Mediterranean Diet**	**Typical American Diet**
Beef Fat, Bunge	3	106.3
Corn Oil		
Butter, Anhydrous	16	34
Lard		
Flaxseed Oil	7	
Menhaden Oil, ARBP-F	10	
Olive Oil	107	
Safflower Oil		12
Soybean Oil		
Total	**143**	**152.3**
Fatty Acid Profile	**Mediterranean Diet**	**Typical American Diet**
C2, Acetic	0.0	0.0
C4, Butyric	0.5	1.1
C6, Caproic	0.3	0.6
C8, Caprylic	0.2	0.4
C10, Capric	0.4	0.9
C12, Lauric	0.4	1.0
C14, Myristic	2.4	6.6
C-14:1, Tetradecanoic, Trans	0.1	0.4
C14:1, Myristoleic, n-9	0.3	1.1
C15	0.1	0.5
C16, Palmitic	19.0	34.8
C-16:1, Hexadecenoic, Trans	0.1	0.6
C16:1, Palmitoleic, n-9	2.7	3.8
C16:2, n-4	0.2	0.0
C16:3, n-9	0.2	0.0
C16:4, n-4	0.2	0.0
C17	0.1	1.5
C17:1	0.0	0.8
C18, Stearic	5.5	26.1
C18:1, Oleic, n-9	82.7	48.1
C18:1, Elaidic, Trans	0.5	6.8
C18:1, n-7, Vaccenic	0.0	0.0
C18:2, Linoleic	15.7	13.3
C18:2, Octadecadienoic, Trans	0.1	0.8
C18:3, Linolenic	4.9	0.7

C18:3, n-6	0.0	0.0
C18:3, Trans	0.0	0.1
C18:4, Stearidonic	0.3	0.0
C19, Nonadecanoic	0.0	0.0
C20, Arachidic	0.6	0.5
C20:1	0.4	0.2
C20:2	0.0	0.1
C20:3, n-6	0.0	0.1
C20:3, n-3	0.0	0.0
C20:4, Arachidonic, n-6	0.2	0.0
C20:4, n-3	0.0	0.0
C20:5, Eicosapentaenoic, n-3	1.4	0.0
C21, Heneicosanoic	0.0	0.4
C21:5, n-3	0.1	0.0
C22, Behenic	0.0	0.0
C22:1, Erucic	0.0	0.0
C22:4, Clupanodonic, n-6	0.0	0.0
C22:5, Docosapentaenoic, n-3	0.3	0.0
C22:6, Docosahexaenoic, n-3	1.0	0.0
C24, Lignoceric	0.1	0.0
C24:1	0.0	0.0
Total	**140.9**	**151.1**

Fat Types	**Mediterranean Diet**	**Typical American Diet**
Saturated (g)	29.5	74.4
Monounsaturated (g)	86.2	54.0
Polyunsaturated (g)	24.4	14.1
Fat %kcal		
Saturated (%)	20.9	49.3
Monounsaturated (%)	61.2	35.7
Polyunsaturated (%)	17.3	9.4
n6	15.9	13.4
n3	7.9	0.7
n6:n3 ratio	2.0	19.0
trans fat (gm)	0.8	8.6

Table 8. Fatty Acids in the Experimental Diets.

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VITA

PERSONAL BACKGROUND

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EDUCATION

Doctor of Philosophy, Experimental Psychology, Texas Christian University, Fort Worth, Texas, 2023
Master of Science, Experimental Psychology, Texas Christian University, Fort Worth, Texas, 2020
Bachelor of Arts, Psychology, Vanguard University of Southern California, Costa Mesa, California, 2017
Diploma, Foothill High School, North Tustin, California, 2013

EXPERIENCE

Teaching Assistant, Texas Christian University, 2018–2023

Instructor, Introductory Neuroscience, Texas Christian University, 2022

Laboratory Instructor, Texas Christian University, 2018–2020

Research Student, Department of Psychology, Vanguard University, 2013

Research Fellow, Department of Psychology, Vanguard University, 2012

VOLUNTEER WORK

President of *Psi Chi*, Vanguard University Chapter, 2013

PROFESSIONAL MEMBERSHIPS

Society for Neuroscience, 2019–present

Psi Chi Psychology Honors Society 2011–present

ABSTRACT

FOOD FOR THOUGHT: EXPLORING MULTIGENERATIONAL EFFECTS OF A COMPREHENSIVE MEDITERRANEAN DIET ON COGNITION AND BIOMARKERS OF ALZHEIMER'S DISEASE

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Recent evidence suggests that approximately 40% of all dementia cases, including Alzheimer's disease (AD), could potentially be prevented by targeting modifiable risk factors, like mid-life obesity (Livingston et al., 2020). Thus, it is crucial to understand how nutritional prevention strategies, like early-life implementation of the Mediterranean Diet (MD), could potentially reduce risk factors and prevent AD later in life. Notably, a recent study found that strict adherence to the traditional MD was associated with a 72% reduced risk of dementia in older Greek adults (Charisis et al., 2021). Although *individual* Mediterranean dietary factors have been shown to protect the brain from AD neuropathology and cognitive impairment in rodents, the biological and behavioral mechanisms underlying a *comprehensive* MD warrant further investigation. Therefore, the current experiment investigated the potential, protective effects of a comprehensive MD compared to a macronutrient-matched typical American diet (TAD) on AD markers in C57BL/6 mice. Following six months of diet administration, male mice on the MD had reduced body weight, adipose fat, fatty liver, serum TNF-α, cortical and hippocampal $A\beta_{1-42}$, and improved spatial memory compared to males on the TAD. Interestingly, although both diets were macronutrient-matched, males on the MD consumed more kilocalories per day compared to males on the TAD. In females, the MD prevented elevated cortical $A\beta_{1-42}$, serum TNF-α, and fatty liver but had no impact on kilocalorie intake, body weight, adipose fat, or spatial memory. Additionally, the MD suppressed LPS-induced neuroinflammation and BDNF

loss in males. Behavioral changes, including increased exploratory activity and reduced anxiety, were observed in males on the MD, but not in females on the MD. Our findings in male mice supported our hypothesis that the MD would play a protective role against AD-related physiological, biological, and behavioral alterations compared to the TAD, and also emphasized the importance of diet composition rather than caloric intake alone.