THE EFFECT OF HIGH-FAT, WESTERN DIET VS. PLANT-BASED, MEDITERRANEAN DIET ON ALZHEIMER'S PATHOLOGY IN MICE

by

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Submitted in partial fulfillment of the requirements for
Departmental Honors in the Department of Biology
Texas Christian University
Fort Worth, Texas

May 4, 2020

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ABSTRACT

Every 65 seconds, someone is diagnosed with Alzheimer's Disease (AD). Individuals suffering from AD experience both behavioral and psychological symptoms, such as cognitive decline, memory deficits, and confusion. Two hallmark pathologies of AD include amyloid-beta (Aβ) and inflammation. The peptide Aβ is derived from the amyloidogenic cleavage process of amyloid precursor protein (APP). Although inflammation is a natural process induced by the innate immune response, chronic inflammation can render serious consequences that contribute to the development of AD pathologies. Simultaneously, the accumulation of Aβ and chronic inflammation can lead to neurodegeneration, neuronal death, and cognitive dysfunction. As AD prevalence rapidly increases, researchers are investigating potential therapeutic interventions that may attenuate AD pathologies and cognitive decline. Some notorious risk factors for AD include insulin resistance and obesity, and accumulating evidence also suggests that dietary factors play a pertinent role in AD development. Prior research has found that several components of the Western diet, including saturated fatty acids, refined sugar, and animal product consumption, are associated with higher AD prevalence. Conversely, plant-based diets, such as the Mediterranean diet (which contains low quantities of saturated fatty acids and high quantities of polyunsaturated fatty acids and monounsaturated fatty acids) have demonstrated therapeutic potential against AD pathologies. Therefore, the current study hypothesized that long-term consumption of a plant-based, Mediterranean diet would reduce cognitive decline induced by Aβ accumulation and inflammation in C56BL/6J mice. At two months of age, mice were assigned to one of the following diet conditions: a plant-based, Mediterranean diet, the Western diet, or a standard (control) diet for four months. Following diet consumption, mice underwent behavioral testing. Our laboratory has utilized lipopolysaccharide (LPS) in previous experiments to induce an

immune challenge that increases soluble Aβ and cognitive deficits which resemble AD development. Mice were treated with LPS or sterile saline and subject to contextual fear conditioning to measure cognitive deficits induced by LPS following long-term diet consumption. We hypothesized that the plant-based, Mediterranean diet would protect mice against cognitive deficits and AD-like pathologies induced by LPS treatment, in comparison to the Western diet.

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ACKNOWLEDGMENTS

I am so grateful to TCU for the opportunity to conduct undergraduate research and am thankful for my wonderful and supportive committee members, Dr. Boehm and Dr. Curtis. It was an unbelievable blessing to work side by side with them and their graduate students. While I worked on my honors thesis, the graduate students pursued their masters and PhDs. They were not only extremely helpful due to their brilliance, but unbelievably inspiring as well. I am so thankful for Paige Braden's wisdom and guidance throughout the construction of this research and writing. Paige became my role model the moment she arrived on campus and her kindness, brilliance, and adaptability shaped my expectations for the kind of doctor I want to become.

I owe most of what I've learned and 99% of my belly aches from laughing too hard to Dr. Chumley. Over the course of a 1-hour meeting at the beginning of my sophomore year, we became fast friends and I wanted to join his lab team almost instantly. Luckily, as I left his office, he shouted: "Hey just let me know when you're ready to join our lab!" I believe that I emailed him the same afternoon. Dr. Chumley is inspiring on every level and I am so thankful for the joy he brings to school. He made research feel as if we were archeologists on the cusp of unearthing a ground-breaking discovery. I'd never considered a career in research until I saw through Dr. Chumley how much fun it could be! Hopefully one day I will have a lab of my own and will bring everything I learned from him with me.

I am most grateful for my family, who without any science background, engaged with me through hours of phone calls about the new things I learned in the lab. For over a decade they woke up before sunrise to drive me to swim practice and travel all over the country so I could compete at swim meets; I am so thankful for their sacrifices so that I could receive an athletic

scholarship in order to attend TCU. Through all of the teary FaceTime calls and "I-don't-think-I'm-cut-out-for-this" moments… they have kept this ship afloat.

I would like to thank my savior, Jesus Christ for giving his life to save mine. God made a way for me, even in my brokenness, to live a life that is purposeful and full of meaning. I am so thankful for His perfect plan for me, even though organic chemistry at TCU made me question it at times! I am thankful for the opportunity to serve others in His name because He first loved and served me. Lastly, I am so blessed for the opportunity to share the gospel on campus and live in the joy and hope that comes from walking with Jesus.

INTRODUCTION

Alzheimer's Disease (AD) is currently the 6th leading cause of death in America and it is estimated that by 2019, roughly 5.8 million Americans had received an AD diagnosis. The disease disproportionately affects women, with nearly twice as many women afflicted in comparison to their male counterparts. Additionally, numerous studies have shown that age is one of the most prolific risk factors for the development of AD—one in three individuals ages 65 and above are affected (Alzheimer's Association). Unfortunately, the statistics concerning the escalation of the disease are not stagnant. In the year 2035, when the entire Baby Boomer generation is 65+ years old, older Americans will comprise 21% of the population, elevated from 15% in 2019 (Nasser, 2019). The "Graying of America" poses a huge threat not only to the American economy but will have an immense impact on health care. Less than ten years ago, the risk factor for developing the disease at 65+ was 0.4%; in 2019, the risk factor increased to 1 in 10 adults aged 65+ (Alzheimer's Association, 2019). As the number of people afflicted with AD rises, it will become paramount to secure effective treatment options.

Conceiving treatment protocols for AD has proven quite difficult: the causes of AD are challenging to confirm due to environmental risk factors and comorbidity. Fortunately, the pathology of the disease has been previously established (Alzheimer's Association; Selkoe, 2004). Amyloid precursor protein (APP) is cleaved into smaller, sticky amyloid-ß (Aß) fragments that clump together in the hippocampus and temporal lobe regions. When these Aß plaques cannot be cleared from the brain they accumulate and disrupt synapses, thus killing neurons. Tau is another important protein implicated in the development of AD. In the healthy brain, tau aids in cellular transport of nutrients in the brain and reinforces microtubules. In the AD brain, hyperphosphorylated tau causes neurofibrillary tangles that disrupt this system and

promote neuronal death. In addition to the contribution of cellular components in the progression of AD, inflammation also plays an important role in the establishment of disease pathology. The brain degeneration found in AD patients is not caused solely by the formation of plaques and tangles but also results from environmental stressors afflicting chronic inflammation on the body (National Institute on Aging, 2019). The inflammatory cascade that results in response to brain degeneration is a clear indicator of disease progression and causes damage through microglial activation, thus weakening the body's immune response.

Though the scientific community is in consensus that AD is a comorbid disease with far-reaching roots, possessing a family history of AD greatly increases the risk of developing the disease. (Alzheimer's Association, 2019). Most genetic factors that have been previously established cause the formation of senile plaques in the brain. Senile plaques are deposits of Aß protein due to neurodegeneration and aging. Even though acquiring these heritable genes places patients at a near-guaranteed risk for developing the disease, it is estimated that less than 1% of diagnosed cases are due to genetic factors (Alzheimer's Association). As a result, AD is primarily understood as a "comorbid" disease having many contributing causes which are also implicated in the development of the pathologies of other diseases.

In recent studies, research has shown that high blood pressure and lack of exercise contribute significantly to the development of AD pathology (Alzheimer's Society; Cass, 2017). As such, participating in exercise could significantly reduce an individual's likelihood of an AD diagnosis (Bernardo, 2016; Morris, 2017). The numerous benefits in daily exercise cannot be overstated—exercising improves blood flow to the brain to help oxygenate the tissue and keep the brain healthy. Exercise has also been shown to increase hippocampal volume; the hippocampus is the region in the brain that is responsible for learning and memory and is injured

in AD patients (Cass, 2017). Elevated blood pressure could be partially responsible for the memory loss seen in AD patients due to damage to the blood vessels of the brain. Additionally, high blood pressure increases oxidative stress, inducing an inflammatory response in the brain—both of which are known contributors to the development of AD pathology (Feldstein, 2012).

Though some risk factors seem unavoidable, numerous conditions contribute to the formation of Alzheimer's pathology and many can be combatted by alteration of the patient's environment or habits. Especially in older populations, researchers have discovered that increased intellectual activity and the formation of social connections may be neuroprotective. The functional capacity of the brain has also been linked to adequate amounts of sleep and decreased stress, both of which ameliorate inflammation in the brain, delaying the onset of AD pathology (Lupien, 2007; Vyas, 2016). The present study focuses on the effect that diet has on the formation of AD pathology in the hippocampus and cortex. Regrettably, we live in a weight loss crazed society where the goal of most diets is to beautify the exterior rather than the inside of our bodies; more attention is owed to the *quality* of the food we consume and the effect that food has on the brain. Since patients may have some control over these factors, emphasizing healthier eating habits could delay or prevent the development of AD.

Past decades labeled fat as the enemy in the American diet and during the 1980's, "low-fat" items became increasingly popular in grocery stores. Since then, nutrition has become an increasingly hot topic as obesity and cardiovascular issues continue to skyrocket. Carbohydrates have become the enemy in recent years and American's have reverted to a high-fat diet to avoid weight gain at the hands of the quickly digestible carbohydrates we consume. Diet fads such as Atkins and the Ketogenic diet are common and have obtained a cult following. While excluding carbohydrates is not usually beneficial for the body except for weight loss, we know that when

consumed in excess, the body has more sugar than necessary for fuel and consequently stores the energy in the form of fat. Unfortunately, following this switch in dietary focus from fats to carbohydrates, the general public has not shown concern about the quality of fats they consume. High-fat diets (especially rich in saturated and trans fats) have been shown to cause diabetes and heart disease through the development of low-grade, systemic inflammation in the body (Gepner, 2019; Nie, 2020; Smith, 2020). Inflammation introduced to the body as an effect of the high-fat diet creates an environment in the brain that leaves the patient more susceptible to AD (Kothari, 2017). Specifically, inflammation alters brain insulin signaling which leads to disruptions in cognitive function. Because of the inflammatory properties associated with fat consumption, long term exposure to the high-fat diet increases the amount of Aß plaque deposition in the brain (Busquets, 2017). In conclusion, neurodegeneration can occur directly as a result of the quality of the diet or comorbidly with diseases such as obesity, which is associated with increased brain age from midlife.

In addition to changes in insulin signaling, there are numerous alterations that occur in the brain due to the effects of the high-fat diet. Foods that are high in fat, which also generally contain high-sugar content, are implicated in the formation of the bliss point. To foster the desire in consumers to eat more and more of their product, the food industry in the United States has engineered the ratio of salt, sugar, and fat in foods to make it nearly irresistible. The bliss point achieved through this carefully calculated ratio triggers the brain's reward center, causing dopamine release. Dopamine causes intense cravings associated with energy-rich food: those which are high in fat and sugar (Volkow, 2017). The reward system for indulging in these foods downregulates the dopaminergic response associated with healthier eating habits. Once the bliss point has been reached, other mechanisms of regulating intake (such as the release of insulin and

leptin) are inhibited (Centers for Disease Control and Prevention, 2019; Koch, 2019). This effect has been observed in both humans and mice. The presence of Reactive Oxygen Species (ROS) and oxidative stress due to the consumption of the high-fat diet causes increases in inflammation. Inflammation is not only damaging to the brain but has also been shown to induce insulin resistance (Freeman, 2013; Matsuzawa-Nagata, 2008; Pistell, 2010). Insulin resistance occurs primarily in response to unhealthy dietary and exercise habits. The inflammatory response that develops due to the consumption of a high-fat diet also decreases the body's sensitivity to leptin secretion (Woods, 2004), which is a hormone involved in hunger suppression. As a result of decreased sensitivity, the sensation of satiety is never reached, resulting in over-consumption. Tragically, the increased release of leptin causes even more inflammation in the brain and triggers microglial activation in response to the damage in the brain (Luna, 2020; Stein, 2020).

Withholding slight variations due to geographic and economic resources, many of the components of the traditional Western diet are consistent across cultures and have been carefully dissected in recent years as obesity levels continue to rise (Engin, 2017; Saltiel, 2017; Solfrizzi, 2005; Wanrooy, 2018). One of the most critical components of healthy fat consumption is the ratio of polyunsaturated fatty acids (PUFAs) to monounsaturated fatty acids (MUFAs) (DiNicolantonio & OKeefe, 2019; Simopoulos, 2016). The most abundant dietary PUFA and MUFA is omega-3 fatty acid and omega-6 fatty acid, respectively. Both omega-3 and omega-6 fatty acids must be consumed through the diet: the body is unable to produce or convert precursors into these fatty acids (National Institutes of Health, 2019). While omega-6 can be a good replacement to the fats in animal products (Mayo Foundation for Medical Education and Research, 2019), subscribers to the Western diet typically overindulge in omega-6 in comparison to the amount of omega-3 fatty acid they consume (Cândido, 2018). Consumers of the Western

diet typically consume a 15:1 ratio of omega-6 to omega-3 fatty acids (Simopoulos, 2002). Omega-6 fatty acids are found in vegetable and corn oil, which are common cooking ingredients among Western cultures (Harvard Health Publishing, 2009). Though omega-6 has been shown to have anti-inflammatory properties and decrease harmful LDL cholesterol, too much omega-6 fatty acid in the diet can cause blood clots and lead to heart attack and stroke (Harvard Health Publishing, 2009; Patterson, 2012).

In addition to the oil content in the Western diet, animal products occupy a large portion of the typical plate. Animal products have high-protein content which is essential for muscle repair and growth, but overconsumption of protein is a hallmark of the Western diet and can cause disruptions in renal function and bone strength (Delimaris, 2013). Additionally, milkfat and casein are commonly implicated in the development of inflammation in the body. Sugar is yet another highly inflammatory component of the Western diet and is typically found in much smaller quantities in other diets (Beilharz, 2016). The detriment of systemic inflammation due to diet is far-reaching, even having prolific effects on the brain, which will be evaluated in this paper.

In complete contrast to the Western diet, numerous aspects of the Mediterranean diet make it an ideal source of fuel for the body. The Mediterranean diet contains many antioxidants (such as selenium and vitamin E found in nuts) which combat the damage inflicted upon cells by other forms of stress on the body (Billingsley & Carbone, 2018; Visioli & Galli, 2001). Some of the most prevalent ingredients in the Mediterranean diet are soy protein, olive and fish oil, choline, and vitamins A, D3, E, C, FA, and B12. Soy protein has been shown to increase cardiovascular health, whereas the protein sourced from the Western diet comes at a cost for the cardiovascular system (Li, 2020; Mozaffarian, 2016). Olive and fish oil have a better balance of

omega-6 and omega-3 fatty acids (roughly 1:1) than vegetable oil and can increase heart health (Simopoulos, 2002). Choline is important for the metabolism and the formation of cell membranes. Like vitamins A, E, and B12, choline must be obtained through the diet and is not synthesized in the body in high enough amounts to generate the required benefit for the body (National Institutes of Health, 2020). Geographically, there is less prevalence of Alzheimer's Disease in regions of the world where the Mediterranean diet predominates culturally. Because these areas are typically surrounded by coasts, they are called "blue zones."

One significant aspect of the Mediterranean diet which was a focus during this study is the impact that diet has on the growth and protection of the microbiome in the gut. The low pH in the colon, which limits the growth of harmful bacteria, and the high fiber content of the Mediterranean diet are jointly responsible for balancing the pH of the gastrointestinal (GI) system. The acidity of the GI system influences which kinds of bacteria can colonize the gut; some microbes are more sensitive to lower pH environments than others (Holscher, 2017). Before having access to the extensive microbiome research that has been conducted in recent years, most people suspected that our microbes were dangerous because they were so poorly understood. In fact, the microbiome was not even known to exist until the late 1990s. Shortly after the microbiome's discovery were monumental developments in science with far-reaching impacts on many fields— especially immunology. The immune system plays a critical role in the development of AD pathology and is now understood to be heavily influenced by the health of the microbiome (Gray, 2020; Ciccocioppo, 2020; Dursun, 2015). Interestingly, the number of bacteria in the microbiome outnumber human cells ten to one. With such a presence in the human body, it is no wonder that scientists care quite a bit about the effect these inhabitants are having on our bodies. Though some microbes in the gut are pathogenic and potentially harmful,

it is possible to crowd out pathogenic bacteria by having an army of healthy microbes which serve as a protective barrier to the rest of the body systems. Remarkably, the microbiome is now considered a supporting organ.

Unfortunately, but perhaps not surprisingly, the microbiome is significantly impacted by poor changes to our diet. Diseases like diabetes and obesity are incredibly harmful to the gut and destroy the protective function of the microbiome through the development of chronic inflammation (Duan, 2018). Curiously, negative alterations in the gut microbiome may even contribute to Aß deposition in the brain. In shocking contrast, the presence of a healthy gut microbiome helps stimulate the immune system (Smith, 2013). Additionally, some microbes in the intestines break down toxic food compounds and prevent them from being absorbed into the bloodstream while other microbes have even been found to generate vitamins that the body needs (Claus, 2016; LeBlanc, 2013). In this way, the relationship we have with the microbes is mutualistic in nature. Though it seems unlikely that the microbiome would have a significant impact on the brain far from its home in the gut, the neuroprotective contribution of these microbes cannot be denied (Proctor, 2017)!

MATERIALS AND METHODS

Experimental Subjects

WT C57BL/6J male and female animals were housed in groups of 3–4 in standard polycarbonate mouse cages (30 x 20 x 16 cm) at ambient temperature (22 °C). Following weaning at one month of age, animals were habituated in a new study room for one month and were allowed access to the standard chow diet (LabDiet, Prolab RMH 1800-5LL2; St. Louis, MO) and water ad libitum. The lights in the study room were set to an automated 0700 on and 1900 off light-dark cycle. All behavioral testing was conducted between 7:30 and 9:30 a.m. All animals received care consistent with the Guide for the Care and Use of Laboratory Animals and the experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee at Texas Christian University.

Figure 1. Experimental Timeline. At 2 months of age, animals were placed into three dietary groups for 4 months. Open field behavior was assessed prior to the last week of the study. LPS was then administered once per day during the final 7 days and was followed by contextual fear conditioning. Finally, animals were euthanized and tissue collected for downstream assays.

Experimental Diets and Treatment Conditions

At two months of age, mice were assigned to one of three diet conditions: a plant-based Mediterranean diet, a high-fat Western diet, or continuation of the standard (control) diet. All diet groups were able to consume their respective chow *ad libitum*. The Mediterranean diet was

formulated in collaboration with Dr. Jada Willis from the Department of Nutrition at TCU and the nutrition team at Teklad Laboratory Animal Diets (Harlan Teklad, Madison, WI) and consists of primarily plant-based ingredients, with the exception of fish oil, which is a key component of the Mediterranean diet. The standard Western diet chow used in this study was designed and synthesized by Teklad (Teklad Custom Diet; TD.88137). The control diet used in this study is a standard rodent chow made by LabDiet. Animals consumed their assigned diets for four months and then completed behavioral testing. Small, ceramic bowls were placed in each study cage to hold the specially formulated rodent chow.

Open Field Test

Before the administration of LPS or saline injections, we measured exploratory and anxiety-like behaviors in mice using open field behavioral testing during the last week of diet consumption. Four open field maze chambers (27 x 27 cm) were used for testing while video tracking software was used to measure each animal's activity (Med Associates Incorporated, St. Albans, VT). Prior to testing, we established two zones to measure the amount of time spent in the center zone versus the outer zones. The center of the chamber was designated as Zone 1, meaning that Zone 2 included the remaining space in the chamber. The time mice spend in the center zone versus the outer zone is used to measure anxiety-like behavior. For example, more time spent in the center suggests that the mouse is exhibiting less anxiety-like behavior and more exploratory behavior. To conduct testing, animals were removed from their home cages and placed individually in the middle of the open field maze. They were then allowed uninterrupted movement for 10 minutes. Following testing, animals were placed back in their home cages. We measured four dependent variables. First, we measured the duration of time in the center zone and duration of time spent rearing (vertical counts), each of which indicates exploratory

behavior. We also measured the total ambulatory distance (cm) traveled throughout the entire 10-minute testing period, in addition to the average speed of the mice.

Inflammation Model

Animals underwent seven consecutive days of once-daily injections of LPS at 250 µg/kg of body weight (*Escherichia coli* serotype 055: B5; Sigma Aldrich, St. Louis, MO) or a standard 200µL of sterile, phosphate-buffered saline (Dulbecco's PBS; Caisson Laboratories, Smithfield, UT) after four months of diet consumption.

Contextual Fear Conditioning

After the week of injections, all groups participated in contextual fear conditioning (CFC). CFC is a behavioral test that utilizes an adverse stimulus during training (day 1) to assess learning in the testing phase (day 2). During the training session, one mouse was placed in the CFC apparatus for 120 seconds before receiving a foot shock. Following the application of the adverse stimulant, the mouse remained in the apparatus for an additional 60 seconds. Immediately after training, all mice were placed back in their home cages. On testing day (exactly 24 hours later) mice were returned to the CFC apparatus for 120 seconds but did not experience a shock. The 7" x 7" x 12" CFC chamber, made by Coubourn Instruments (Whitehall, PA,) is designed to provide animals with memorable sensory cues—the walls of the container are covered in polka dot wallpaper and peppermint scent is placed under the electric grid. Our laboratory has previously demonstrated that repeated injections of LPS decrease the freezing behavior which is expected during CFC testing. Animals without learning and memory disruption should recognize the sensory cues on testing day and associate the surrounding environment with the foot shock they received the day prior, thus exhibiting freezing behavior. These decreases in freezing behavior following repeated LPS administration suggest that

elevated Aβ and inflammation induced by LPS disrupts learning and memory processes (Kahn et al., 2012). The percent time mice exhibited freezing behavior during this study was computed by FreezeFrame^{TM} software (ActiMetrics Software, Wilmette, IL).

Tissue Collection

The day following the completion of CFC testing, mice were decapitated and the hippocampus, frontal lobe, and dorsal cortex were removed. First, both hemispheres of the hippocampus were collected and placed in Proprep (PRO-PREP, Bulldog Bio, Portsmouth, NH,) which was supplemented with additional protease and phosphatase inhibitors. Second, the frontal lobe was removed and stored in a tissue protein extraction buffer (T-PER; Invitrogen, ThermoFisher Scientific, Waltham, MA) and was supplemented with protease and phosphatase inhibitors. Lastly, the dorsal cortex was collected and placed in the same tissue protein extraction buffer with protease and phosphatase inhibitors as that used for the frontal lobe. All brain tissues were placed on dry-ice following the collection process and were then transferred to and stored in a freezer at -80º C.

$A\beta_{42}$ ELISA

We then used a Mouse Aβ42 ELISA (Invitrogen, ThermoFisher Scientific) to measure soluble $A\beta_{42}$ from the hippocampus. The hippocampi lysates with a protein concentration >200pg/mL were diluted in incubation buffer in a 1:2 dilution ratio. The $A\beta_{42}$ standard (a lyophilized synthetic peptide) was reconstituted with standard reconstitution buffer and made into serial dilutions. Following dilutions, 100 µL of lysates and standards were plated into antibody-coated wells and were incubated at room temperature for two hours. The plates were then aspirated and washed four times with wash buffer (1X). Each well was treated with Aβ42 Detection Antibody solution and incubated for one more hour at room temperature. Following

incubation, the plates were washed again and treated with 100 μL of HRP-tagged detection antibody (Anti-Rabbit IgG). After 30 minutes of room temperature incubation, the plates were washed a sixth time and were treated with 100 μL stabilized chromogen (tetramethylbenzidine). The plates were incubated in a dark environment at room temperature for 30 minutes. Finally, 100 μL 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).

Iba-1 Western Blotting

Microglial activation in the frontal lobe and dorsal cortical regions was measured using an Iba-1 antibody. First, brain lysates were diluted with sample buffer. The lysates were then boiled for five minutes at 100 °C and transferred to ice. Samples (20 μL) were centrifuged and loaded into 4–20% Mini-PROTEAN TGX polyacrylamide gels (BioRad, Hercules, CA). Gels were loaded into an electrode holder and placed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS Page) apparatus containing SDS running buffer. Gel electrophoresis was run at 200 volts, 300 watts, and 3.0 amps for 55 minutes. The gel was then transferred to 0.45μm Hybond PVDF membranes (Genesee Scientific, San Diego, CA) and blocked with 5% BSA (albumin, bovine fraction) in Tris-buffered saline with Tween-20 (BSA; Research Products International, Mount Prospect, IL). Next, the membrane was treated with Iba-1 primary antibody (IBA1 Polyclonal Antibody, ThermoFisher Scientific) and incubated at 4º C overnight. The next day, the membrane was washed in Tris-Buffered Saline and Tween Detergent (TBST) for one hour and treated with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for two hours. Finally, the membrane was washed in TBST for one hour and treated with chemiluminescent solution (SuperSignal West

Pico PLUS Chemiluminescent Substrate, ThermoScientific, Waltham, MA), and imaged (G:Box Chemi ST 4, Syngene, Frederick, MD).

Statistical Analyses

All data were analyzed with Statistical Package for Social Sciences (SPSS; Version 23.0, IBM, Armonk, NY). The experimental design of the current study consisted of two, 3 (diet condition: Mediterranean diet, Western diet, vs. control diet) by 2 (treatment condition: LPS or saline) analyses of variance (ANOVA). Multiple ANOVAS were conducted to examine the dependent variables of both behavioral and biological measures. Before running the statistical analyses, the Shapiro-Wilk's test was used to assess normality. An alpha level ≤ 0.05 was considered significant in all statistical analyses. However, if the assumption of normality was violated, a more conservative alpha level, ≤ 0.01, was adopted. Finally, to examine interactions, posthoc tests were conducted using Fisher's LSD correction.

RESULTS

Male Weight Data

All mice were weighed once a week from weaning until the conclusion of the experiment to ascertain the effect of the experimental diets on body weight. The first month of weigh data did not reveal a significant difference between experimental groups; this result was expected since all animals consumed the standard, control diet. Using a repeated-measures ANOVA, we discovered a significant main effect of diet condition ($p \leq 0.001$) on body weight (Figure 2A). A posthoc test revealed a significant difference between the Western diet and control diet ($p \leq 0.001$) during each month of the administration of the diet. According to the results of the posthoc test, there was also a significant difference between the Western diet and Mediterranean diet during months two, four, and five of diet consumption ($p \leq 0.001$) and at month three ($p \leq 0.003$). We found that five months of Western diet consumption significantly increased body weight in males in comparison to those consuming either the Mediterranean or standard diet for male mice. Additionally, there was a significant difference between the Mediterranean diet and the control diet ($p \leq 0.024$) at two, four, and five months of diet consumption; however, there were no significant differences between the Mediterranean diet and the control diet ($p > 0.50$) at the three-month mark.

Female Weight Data

Like the male group, the first month of weigh data for females did not reveal significant differences between experimental groups as was expected. Following diet administration, we discovered a significant main effect of diet condition in females ($p \leq 0.001$, Figure 2B) using a repeated-measures ANOVA. A Posthoc tests using Fisher's LSD revealed a significant difference between Western diet and control diet ($p \leq 0.001$) and the Western diet and the

Mediterranean diet ($p \leq 0.001$) at 2 months of diet consumption. Therefore, we were able to conclude that just one month of Western diet consumption significantly increased body weight in females in comparison to the other diet conditions. We found that there was also a significant difference between the Western diet and the Mediterranean diet for females after three months of diet consumption ($p \leq 0.023$) and after four months ($p \leq 0.001$). Additionally, there was a significant difference between the Western and control diets ($p \leq 0.001$) after four months of diet consumption. At five months of diet consumption, we found a significant difference between all diet conditions for female mice ($p \leq 0.001$).

Open Field Test: Males

A one-way ANOVA revealed a significant main effect of diet condition for the total amount of time spent in the maze ($p = 0.004$), total vertical counts ($p = 0.001$), and total distance traveled ($p \leq 0.001$, Figure 4). Additionally, we found a significant difference between the Western diet and control diet ($p = 0.025$), as well as the control diet and the Mediterranean diet ($p = 0.001$) for the total amount of time spent in the center of the maze. For the total vertical counts, there was a significant difference between the Western diet and the Mediterranean diet ($p = 0.001$), as well as the control diet and the Mediterranean diet ($p = 0.004$). Additionally, the difference between the control diet and the Western diet for vertical counts was approaching significance ($p = 0.086$). For the total distance traveled during testing for male mice, we found a significant difference between the Western diet and control diet ($p \leq 0.005$) and a significant difference between the Western diet and the Mediterranean diet ($p \leq 0.001$). We did not find a significant main effect of diet condition ($p = 0.11$) for average speed; however, there was a significant difference between the average speeds of mice consuming the Western diet versus the Mediterranean diet ($p = 0.038$).

Open Field Test: Females

A one-way ANOVA revealed a significant main effect of diet condition for the average speed of animals during testing ($p = 0.001$) but a non-significant main effect of diet condition for total time spent in center ($p = 0.226$), total vertical counts ($p = 0.25$), and total distance traveled ($p = 0.11$, Figure 5). We were, however, able to find that the difference between the Western diet and the control diet was approaching significance ($p \leq 0.058$). We found a significant difference between the averages speeds of mice consuming the Western diet and the Mediterranean diet ($p = 0.001$), as well as between the control diet and the Western diet ($p = 0.016$).

CFC Males

To assess the effect of diet and LPS treatment on learning during contextual fear conditioning, we conducted a 3 (Diet Condition: Mediterranean diet vs. Western diet vs. control diet) x 2 (Treatment Condition: LPS or saline) ANOVA. We found a significant main effect of treatment condition ($p < 0.05$) and a non-significant main effect of diet condition ($p > 0.05$) using a one-way ANOVA (Figure 3A). There was a significant difference in freezing behavior for animals treated with LPS in comparison to those treated with saline for all treatment conditions. Surprisingly, there were no significant differences in the percentage of freezing behavior between diet conditions and a posthoc tests revealed that there were no significant differences between groups ($p > 0.05$).

CFC Females

A one-way ANOVA revealed a significant main effect of treatment condition ($p < 0.05$) and a non-significant main effect of diet condition ($p > 0.05$) in the female mice (Figure 3B), which was consistent with the results obtained for the males. Additionally, posthoc tests revealed that there were no significant differences between groups ($p > 0.05$); however, the difference

between the control diet group and the Mediterranean diet group for the saline treatment condition approached significance ($p = 0.057$). In comparison to mice consuming the control diet, female mice eating the Mediterranean diet exhibited a greater percentage of freezing behavior, approaching significance.

DISCUSSION

Given the vast amounts of research implicating the high-fat, Western diet in the development of obesity and increased weight gain, we hypothesized that the mice in the Western diet group would gain more weight than the mice consuming the Mediterranean and control diets. Additionally, we suspected that the mice in the Mediterranean diet group would gain more weight than the control diet, but not such a drastic difference as seen in comparison to the Western diet. We formulated this hypothesis based on the fact that even though the Mediterranean diet is healthier than the Western diet in composition, it is still high in fat and would result in a significant amount of weight gain as a result. We were able to show with both male and female mice that consumption of the Western diet results in a significant amount of weight gain in comparison to both the Mediterranean and control diet groups. Furthermore, the male mice that consumed the Mediterranean diet had significantly increased weight gain in comparison to the control group, as expected. The female Mediterranean diet mice had significantly increased weight gain in comparison to the mice consuming the control diet, but only after five months of diet consumption. We suspect that the sex differences observed based on diet conditions are a result of the soy content in the Mediterranean diet, which has been implicated in dysregulation of sex hormones in males and females (Kurzer, 2002).

Before beginning behavioral testing, we hypothesized that mice in the Western diet group would spend more time in the center zone of the open field arena and more total time exhibiting exploratory, rearing behavior in comparison to the mice in the control diet group. Since exploratory behaviors suggest lower levels of anxiety, which is considered abnormal behavior in mice, we believed that the mice consuming the Western diet would experience learning and behavioral deficits as a result of exacerbated inflammation in their brain. We found that the mice

consuming the Western diet spent significantly more time in the center of the field than the mice on the control diet, as expected. Surprisingly, the male mice consuming the Mediterranean diet also spent significantly more time in the center of the field than the control mice. For the male mice, this result is likely a sex difference based on the metabolism of soy in the Mediterranean diet. Similarly, the mice in the Mediterranean diet group spent more time exhibiting rearing behavior than the male mice on the control diet. We hypothesized that the mice in the Mediterranean diet group would spend the most time of all diet conditions in the periphery of the field and spend the least amount of time exhibiting rearing behavior. Surprisingly, the mice consuming the Western diet spent significantly less time rearing than those consuming the Mediterranean diet which was a likely result of sickness due to the combination of diet condition with the unfortunate infection of our mouse colony with Norovirus and Pinworms (Hsu, 2005).

Following the administration of LPS or saline injections for seven days, we conducted CFC testing. Our literature review leads us to believe that mice in the Western diet group would exhibit less freezing behavior than the mice on the Mediterranean and control diets as a result of having the most learning inhibition following the day of training. Additionally, we hypothesized that the mice consuming the Mediterranean diet group exhibit the most freezing behavior of all groups. While there were no significant differences found for freezing behavior between mice consuming the Western diet and those in the control and Mediterranean groups, the female mice receiving saline injections in the Mediterranean diet group exhibited freezing that was approaching a significant difference when compared to the control group.

Due to complications in obtaining data as a result of the COVID-19 outbreak in the United States, serum results for the samples collected following the conclusion of the experiment have not yet been analyzed. We suspect that upon resumption of this research, we will find an

increased presence of soluble $A\beta_{42}$ from the hippocampus of mice consuming the Western diet (Kahn, 2012) in comparison to mice in both the Mediterranean and control groups. Additionally, we hypothesize that microglial activation, measured by Iba-1 Western Blotting, will also be significantly increased in the mice consuming the Western diet (Navarro, 2018) in comparison to those in the Mediterranean and control groups.

Upon returning to the laboratory, our lab will extrapolate on the findings presented in this study with an additional focus on the composition of the Mediterranean diet. Being that the formulation of the Mediterranean chow was custom designed, we intend to make slight alterations in order to prevent the sex differences we observed during behavioral testing. The ratio of PUFAs to MUFAs will likely be adapted to more accurately represent the Mediterranean diet, thus resulting in less weight gain for the mice consuming this diet. Additionally, the continued use of the seven-day LPS inflammation model is contingent upon the results from the Aβ ELISAs. Though we expect significant findings based on previous studies published from our lab, which have shown that LPS induces elevated levels of Aβ (Kahn, 2012), we have looked into alternative protocols in order to ensure all study animals receive equal doses of LPS.

Ultimately, we hypothesized that the plant-based, Mediterranean diet would protect mice against cognitive deficits and AD-like pathologies induced by LPS treatment, in comparison to the Western diet. Though we were only able to partially complete this study, repetition of these experiments will contribute to our growing knowledge of the relationship between diet and Alzheimer's-like pathology in mice. In support of our original hypothesis, we found that male mice consuming the Mediterranean diet exhibited less cognitive decline than the Western and control diet mice during open field testing. We were also able to establish a significant difference in weight gain for both male and female mice consuming the Western Diet from both the

Mediterranean and control groups, which may establish a relationship between obesity and neurodegeneration in mice.

Figure 2

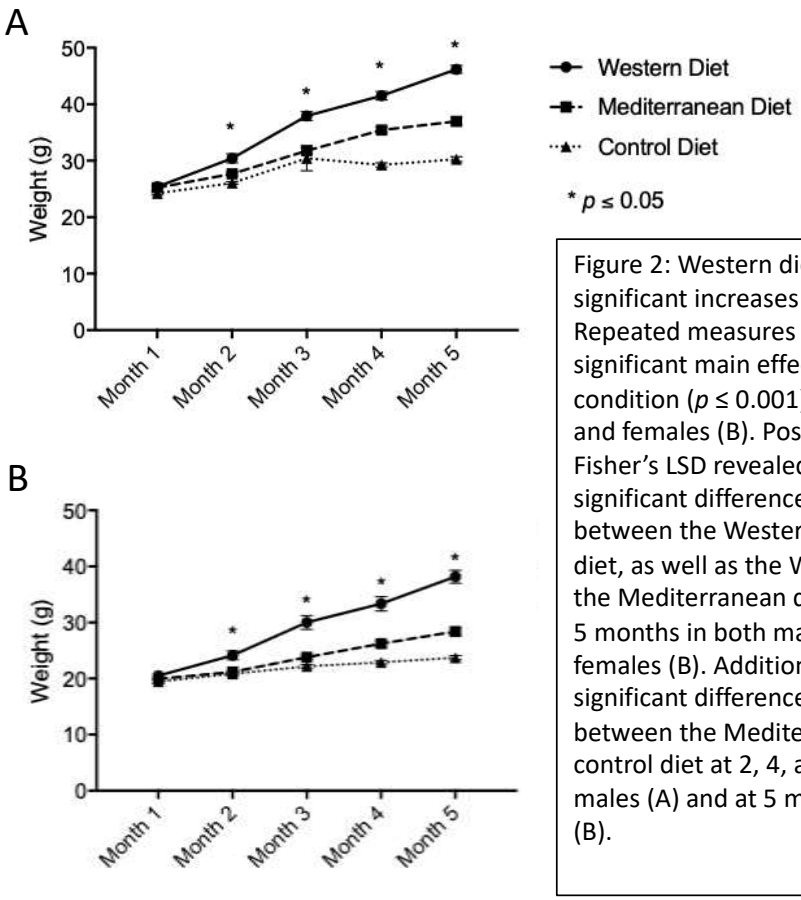


Figure 2: Western diet leads to significant increases in weight. Repeated measures ANOVA revealed a significant main effect of diet condition ($p \leq 0.001$) in both males (A) and females (B). Post-hoc tests using Fisher's LSD revealed that there was a significant difference in weight between the Western diet and control diet, as well as the Western diet and the Mediterranean diet, at 2, 3, 4, and 5 months in both males (A) and females (B). Additionally, there was a significant difference in weight between the Mediterranean diet and control diet at 2, 4, and 5 months in males (A) and at 5 months in females (B).

Figure 3

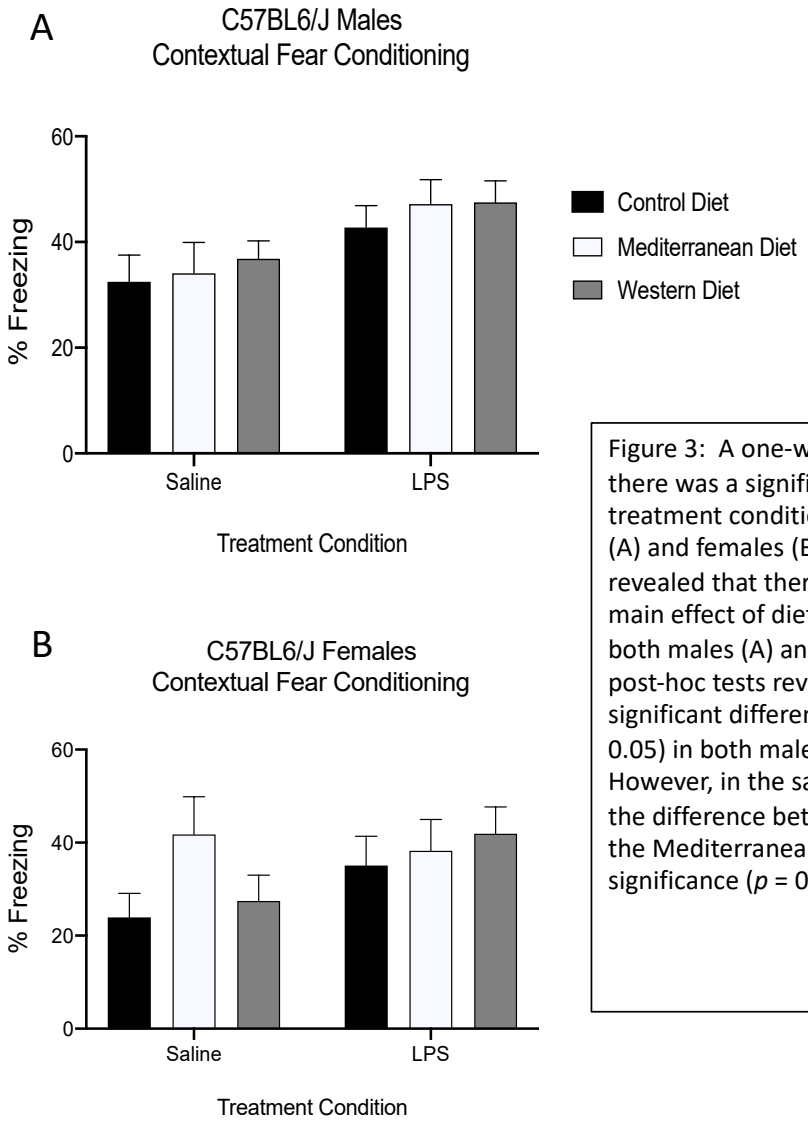
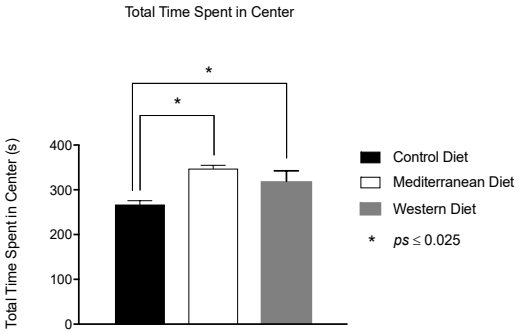


Figure 3: A one-way ANOVA revealed that there was a significant main effect of treatment condition ($p < 0.05$) in both males (A) and females (B). A one-way ANOVA also revealed that there was a non-significant main effect of diet condition ($p > 0.05$) in both males (A) and females (B). Additionally, post-hoc tests revealed that there were no significant differences between groups (ps > 0.05) in both males (A) and females (B). However, in the saline treatment condition, the difference between the control diet and the Mediterranean diet was approaching significance ($p = 0.057$) in females (B).

Figure 4

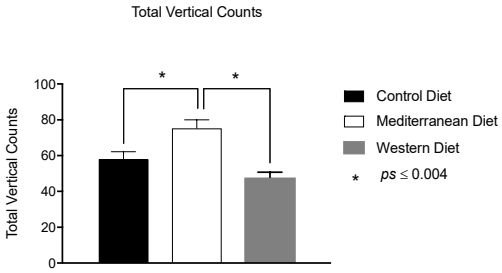
C57BL/6J Males, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a significant main effect of diet condition ($p = 0.004$), and a significant difference between the Western diet and control diet ($p = 0.025$), as well as the control diet and the Mediterranean diet ($p = 0.001$).

n = 20-24

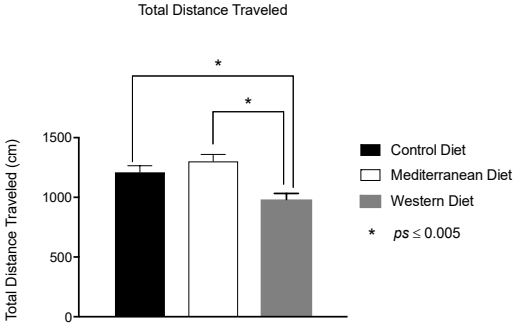
C57BL/6 Males, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a significant main effect of diet condition ($p \leq 0.001$), and a significant difference between the Western diet and the Mediterranean diet ($p \leq 0.001$), as well as the control diet and the Mediterranean diet ($p = 0.004$). Additionally, the difference between the control diet and the Western diet was approaching significance ($p = 0.086$).

n = 24-25

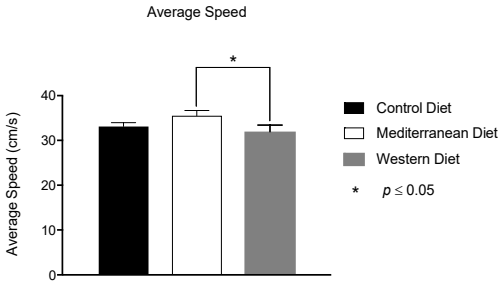
C57BL/6J Males, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a significant main effect of diet condition ($p \leq 0.001$), and a significant difference between the Western diet and control diet ($p \leq 0.005$), as well as the Western diet and the Mediterranean diet ($p \leq 0.001$).

n = 24

C57BL/6J Males, Batches 1 – 3
(6 months old)

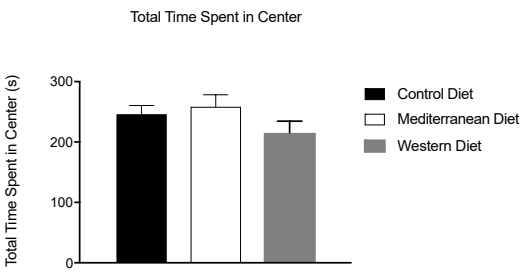


A one-way ANOVA revealed a non-significant main effect of diet condition ($p = 0.11$). However, there was a significant difference between the Western diet and the Mediterranean diet ($p = 0.038$).

n = 24-25

Figure 5

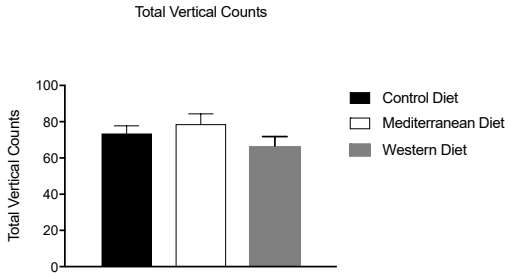
C57BL/6J Females, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a non-significant main effect of diet condition ($p = 0.226$).

n = 21 - 24

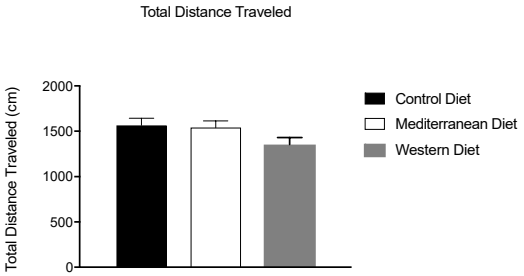
C57BL/6 Females, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a non-significant main effect of diet condition ($p = 0.25$).

n = 24-25

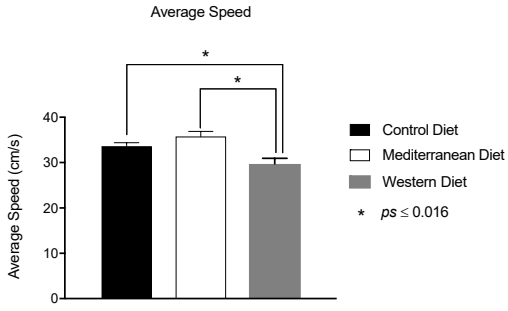
C57BL/6J Females, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a non-significant main effect of diet condition ($p = 0.11$), and the difference between the Western diet and control diet was approaching significance ($p \leq 0.058$).

n = 23 - 24

C57BL/6J Females, Batches 1 – 3
(6 months old)



A one-way ANOVA revealed a significant main effect of diet condition ($p = 0.001$) and a significant difference between the Western diet and the Mediterranean diet ($p \leq 0.001$), as well as the control diet and the Western diet ($p = 0.016$).

n = 24-25

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