# EFFECT OF HIGH DIETARY SUGAR AND METFORMIN IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

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## DEDICATION

……..……..…To My Father……..…….

## ABSTRACT

Alzheimer's disease (AD) starts with toxic changes in the brain disrupting healthy processes and leading to loss of neurons. Dietary sugar can adversely affect health by contributing to inflammation in the body and in the brain (Beilharz, Maniam & Morris, 2015). AD has been referred to as Type 3 diabetes because of the insulin resistance developed in the brain (Kim & Feldman, 2015; Steen et al., 2005; de la Monte, 2009). Conversely, metformin demonstrates beneficial effects, such as anti-inflammatory, increased neurogenesis, enhanced cognition and extended life expectancy (Wang et al., 2012; Saisho, 2015; Martin-Montalvo, et al., 2013). The theory of current study is that disrupted sugar metabolism can create insulin resistance and exacerbate AD pathology. It is hypothesized that high dietary sugar may aggravate AD pathology, while metformin could ameliorate AD. Histology results indicated that sugar increased plaque deposition and inflammation in the brain, while sugar with metformin decreased it.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's disease |
| Aβ | Amyloid-beta |
| APP | Amyloid precursor protein |
| ApoE | Apolipoprotein E |
| ApoE4 | Apolipoprotein E 4 gene |
| ApoE3 | Apolipoprotein E 3 gene |
| Ach | Acetylcholine |
| AChE | Acetylcholinesterase |
| BBT | Balance-Beam Task |
| BMI | Body mass index |
| βAPP | Beta-amyloid precursor protein |
| CR | Calorie restriction |
| CFC | Context Fear Conditioning |
| CFT | Co-factor theory |
| CSF | Cerebrospinal fluid |
| EOD | Early-onset dementia |
| GLP-1 | Glucagon-like peptide-1 |
| HDR | Homology-directed recombination |
| HRSD-17 | Hamilton Rating Scale for Depression |
| IGF-I | Insulin-like growth factor 1 |
| IGF-II | Insulin-like growth factor 2 |
| KO | Knock out |
| KI | Knock-in |
| LOD | Late-onset dementia |
| LTP | Long-term potentiation |
| MCI | Mild-cognitive impairment |
| MADRS | Montgomery-Asberg Depression Rating Scale |
| NFTs | Neurofibrillary tangles |
| NOR | Novel object recognition task |
| PS1 | Presenilin – 1 |
| PS2 | Presenilin – 2 |
| PRS | Polygenic risk scores |
| T1DM | Type-1 Diabetes mellitus |
| T2DB | Type-2 Diabetes mellitus |
| WMS-R | Wechsler Memory Scale Revised |

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1.DEMENTIA

Alzheimer's disease (AD) is a form of dementia. Dementia is a brain disorder associated by major cognitive impairment and brain changes due to many factors. Dementia is not a specific disease, rather it includes many neurodegenerative diseases such as Alzheimer's disease, vascular dementia, Lewy Body disease, head trauma, frontotemporal dementia, Creutzfeldt Jakob disease, Parkinson's disease, and Huntington's disease (Alzheimer Society, 2018). All of these diseases can have similar and overlapping symptoms. Dementia ranges in severity in three stages. First, the mildest stage in which patient experiences functional and cognitive difficulties that were normal before the onset to the moderate stage that includes personality and mood changes, confusion, language and memory problems. In the third stage, the severe stage, patient must depend on caregivers for daily living (National Institute on Aging). Most forms of dementia begin as the result of a loss of synapses and neurons due to the disruption in communication between neurons (Jellinger, 2006; Jackson et al., 2019). Therefore, a person with dementia suffers from a wide range of cognitive and behavioural symptoms.

There are 50 million people worldwide living with dementia, with 10 million new diagnoses every year (Alzheimer's Disease International, 2015). This means that every three seconds someone is diagnosed with dementia each year. According to the World Alzheimer Report (2015), the financial costs of dementia are expected to increase to $2 trillion US dollars by 2030. The cost of care for just one AD patient in the USA is estimated at USD $60,000 annually (Jellinger, 2006). The world-wide cost of formal and

informal care in 2005, based on 29.3 million demented people, was estimated to be USD $315.4 billion (Wimo, Winblad, Jönsson, 2007).

Life expectancy is increasing across the globe, and aging contributes to rapid increase of chronic diseases, including AD. It is estimated that 58 percent of all people suffering from dementia live in economically low income countries, with 4.9 million (49 of total) cases in Asia, 2.5 million (25%) in Europe, 1.7 million (18%) in the Americas, and 0.8 million (8%) in Africa (Alzheimer's Disease International, 2015). It is common for people to have comorbidity of dementias, which means that someone diagnosed with AD can also be living with vascular dementia.

## 1.2.ALZHEIMER'S DISEASE

AD is a neurodegenerative disease and is the most common type of dementia. Importantly for this thesis, AD is linked to inflammation in the brain, due to the insulin resistance developed in the brain, which is associated with impaired cognitive functions (Meraz-Ríos et al., 2013; Akiyama et al., 2000). To appreciate seriousness of dementia, it is recognized as the third leading cause of death, after cardiovascular diseases and cancer (Jellinger, 2006). What causes AD exactly is still largely unknown, but it is known that toxic changes in the brain disrupt healthy brain processes leading to neuronal loss and eventually death. Those who have had first-hand experience with a person living with AD know the progress of this devastating diseases in terms of physical, emotional and financial burden. Gerontologist Dr. Schulz and his colleagues (1995) summarized from many studies that caregivers experience extreme psychiatric and physical difficulties, with depression and anxiety reported at a high rate.

AD is a neurodegenerative disease that disrupts thinking, impairs memory and destroys brain cells with irreversible consequences (Jackson, 2019; Alzheimer Society, 2019). It progressively destroys hippocampus and cortex, causing atrophy of the brain (Verheijen & Sleegers, 2018). AD is defined by development and spreading of Aβ plaques and Tau tangles that are believed to make important contributions to neuronal death and brain shrinkage. Behavioural symptoms in humans include memory loss that disrupts daily functioning, confusion in time and space, problems with writing and speaking, and changes in mood and personality (World Health Organization, 2019).

In 1980, when *Diagnostic and Statistical Manual* (DSM-III) was published, AD for the first time was included as age-related dementia (Jellinger, 2006). The illness lasts, on average, six years (Masters et al., 1985). Between age 65 and 100, the prevalence risk of AD is 33% for men and 45% for women (Jellinger, 2006; Viña & Lloret, 2010). Clearly, AD is more common among females. Most cases of AD are sporadic, affecting about 85 to 95 percent of cases (Meraz-Ríos, Toral-Ríos, Franco-Bocanegra, Villeda-Hernández & Campos-Peña, 2013; McDonald, Craig & Hong, 2010; de la Monte, 2014). Sporadic means that there is not a strong familial or inherited predisposition and it tends to begin after age 65 (Steen et al., 2005). The other kind of AD classification is familial, which relates to known potent genetic mutations affecting 5 to 10 percent of cases and is often referred to as early-onset AD (Meraz-Ríos et al., 2013). It is important to note that nongenetic factors interact with familial type as well.

A causal role in AD for inflammation has been suggested by researchers, however it has been a controversial topic. Some suggest that inflammation in early stages of AD could have a beneficial role, possibly due to the hypothesized role for microglia and

astrocytes in clearing out the Aβ plaques (Meraz-Ríos et al., 2013). Microglia are cells that respond to the injury and occur in the brain (Akiyama et al., 2000). According to several studies, microglia participate in accumulation of Aβ (Meraz-Ríos et al., 2013), while astrocytes are cells in the brain that are involved in the neurotransmitter secretion, recycling, ion homeostasis, metabolism regulation, information processing, signal transmission and modulation of oxidative stress (Halassa & Haydon, 2010).

Although there is no cure for AD, many identified factors contribute to the rate of progression of this neurodegenerative disease. There are several medications that can improve the quality of life for symptoms, such as memory decline, thinking abilities and motor skills (Alzheimer Society, 2019).

At least some of the risk factors for AD are modifiable. It has been suggested that lifestyle factors, such as nutrition, head injuries, excessive stress, and hearing problems can either increase or decrease the risk of AD. Many studies show that more education leads to decreased risk of AD and other types of dementia (Ott et al., 1995). With all the available methods today, AD risk can be identified earlier. These methods include neuropsychology, neuroimaging, and assessments of tau and β amyloid proteins in plasma and cerebrospinal fluid (CSF) (Jillinger, 2006). Mild cognitive impairment (MCI) is the earliest clinical diagnosis in the decline toward AD and is sometimes referred to as prodromal AD.

## 1.3.HISTORY OF ALZHEIMER'S DISEASE

Alois Alzheimer (1864) was a German clinical psychiatrist and neuroanatomist, who on November 3, 1906 in the annual psychiatrist meeting in Tubingen, reported a case about "A peculiar severe disease process of the cerebral cortex". It was a case about a 51

year-old woman named Auguste, who was a patient at the Frankfurt Psychiatric Hospital (Möller & Graeber, 1998; Hippius, 2003). Her mental degeneration was characterized by severe cognitive disturbances, disorientation, delusions, aphasia and unpredicted behaviour. Auguste was admitted to the hospital in March 1901, and on April 8, 1906 she died (Hippius, 2003). After the autopsy, Alzheimer discovered atrophy of the brain (Möller & Graeber, 1998) and histological alterations, which later were known as plaques and tangles (Hippius, 2003). The neurofibrillary tangles were detected in neuron, while senile plaques were discovered in the cerebral cortex (Jellinger, 2006).

In 1908, Kraepelin, who was a very impactful psychiatrist and also Alzheimer's colleague, included the case of Auguste in his textbook *Psychiatrie A* and called it Alzheimer's disease (Hippius, 2003). In 1910, when the book was first published, Alzheimer's disease was introduced to the public and has been used ever since. Meanwhile, Alzheimer's co-worker, Perusini, published a paper in 1910 called *Clinically and histologically peculiar mental disorders of old age*, which included cases identical to the Alzheimer disease (Möller & Graeber, 1998).

In 1911, Alzheimer published the case of Johann F., who was another Alzheimer's disease patient and died three years after hospitalization. Although, with similar symptoms, and the diagnosis of the AD, after histology, Alzheimer found that there were no neurofibrillary tangles in Johann's brain and "plaques only".

When in 1995, Graeber found the material from both Auguste's and Johann's cases and investigated the material with modernized techniques (Hippius, 2003; Möller & Graeber, 1998). It was concluded by Möller & Graeber (1998) that these differences in

plaques and neurofibrillary tangles were because of different stages of the development of AD.

According to Hippius (2003), in 1910, Alzheimer also established a new scientific journal *Zeitschrift für die gesamte Neurologie und Psychiatrie.* Two years later, Alzheimer received his dream job when he was appointed position as a Chair of Psychiatry at the University of Breslau. Unfortunately, he could not devote much time to his research as he passed away at age 51 on December 19, 1915. Later, plaques and amyloid description and relationship between both was first described by Divry (1927), who suggested their common origin (Jellinger, 2006).

## 1.4.EARLY-ONSET DEMENTIA AND ALZHEIMER'S DISEASE

Mostly, people associate AD or dementia with geriatric population, however, AD can affect younger adults as well. Early-onset dementia is referred to anyone who has been diagnosed with it before age 65. It can also occur in people between ages 30 and 65. In a longitudinal study by McMurtray and colleagues (2005), they compared early-onset dementia (EOD) patients with late-onset dementia (LOD) in the sample size of 948 from which 30% of these had an EOD. Although, an important limitation of the study was the fact that the sample collection took place from the Veteran's Affairs Medical Centre with 98 percent dominance in males, they still found some interesting differences between two groups. In particular, EOD patients were mostly affected by the disease due to traumatic brain injuries, alcohol, HIV, and frontotemporal lobar degeneration, including Pick's disease. Conversely, LOD patients were mostly affected by the AD. Vascular dementia affected both groups with equally high percentages. Importantly, researchers found that EOD patients had more treatable and preventable conditions (McMurtray, Clark,

Christine & Mendez, 2005). In most studies, summarized by McMurtray and colleagues, early-onset dementia patients account for 20 to 34 percent with AD. EOD is difficult to diagnose accurately and often clinicians make Type II error, or a false positive, regarding Alzheimer's disease. In other words, they are diagnosed with AD when in fact it is a different form of dementia (McMurtray et al., 2005). Although McMurtray's study did not mention AD as the top diagnosis for EOD, other studies have shown that AD has been identified as the most common form of dementia among young adults as well, specifically early-onset (Harvey et al., 2003; Williams et al., 2001; Yokota et al., 2005).

Researchers have been trying to answer the question of "what is the cause for AD?", but there is no direct conclusion that leads to this answer. Thus far it is known that AD has a genetic predisposition, associated with early onset, as well as sporadic AD. There have been three gene mutations identified that play role in the early onset condition: gene βAPP, presenilin 1 (PS1), and presenilin 2 (PS2) (Rogaeva, 2002; Jellinger, 2007; Verheijen & Sleegers, 2018; Guerreiro et al., 2013, McDonald et al., 2010). These genes impair processing, trafficking and recycling of the amyloid peptide (McDonald et al., 2010). The APP gene is on chromosome 21, the PS1 gene is on chromosome 14, and the PS2 gene is on chromosome 1 (National Institute on Aging). These gene mutations that include APP, PS1, PS2 belong to the familial AD and have been identified to increase amyloid-β production (Chaudhury et al., 2018). This will be discussed in detail in section 1.6. Alzheimer's Disease Pathology.

## 1.5.LATE-ONSET DEMENTIA AND ALZHEIMER'S DISEASE

Late-onset dementia (LOD) refers to people who experience the onset of dementia after age 65. As mentioned before, AD is one of the most common forms of dementia and

in LOD it accounts for 80 percent (Harvey et al., 2003; Verheijen & Sleegers, 2018). With that said, of all the people who have been diagnosed with dementia, 80 percent of them have AD. LOD is based on the same neuropathological features as EOD, which is the amyloid beta deposition and neurofibrillary tangles, however, APP gene mutations have not been identified in the late-onset cases (Schmechel et al., 1993). Late-onset Alzheimer's disease has been associated with Apolipoprotein E (ApoE) gene (Jellinger, 2007, Roses, 2006) with ApoE gene being the most consistent observation associated with AD (Chaudhury et al., 2018). Polygenic risk scores (PRS) are used to predict total risk for an individual to have risk genes in their genome, as well as to predict the risk of LOD from mild cognitive impairment (MCI) (Chaudhury et al., 2018). Especially, with the APOE allele 4 gene (APOE4), which is located on chromosome 19 and has been identified as a risk factor for late-onset AD (Schmechel et al., 1993; Verheijen & Sleegers, 2018; Guerreiro et al., 2013). A Study by Schmechel et al. (1993) compared late-onset AD brains during the autopsy for comparison of APOE4 and APOE3 genes. They discovered that the APOE4 gene of one or two copies present in the brain had increased plaque amyloid deposits compared with APOE3 allele.

## 1.6.ALZHEIMER'S DISEASE PATHOLOGY

Alzheimer's disease is not normal aging; rather, it is a progressive neurodegenerative disease leading to neuron loss and brain shrinkage. AD neuron loss is suggested to be due to increased activation of apoptosis pathways, impaired energy metabolism, mitochondrial dysfunction, chronic oxidative stress and cerebrovascular disease/cerebral hypoperfusion (Steen et al., 2005). This disease is identified by amyloid plaques, consisting of Aβ peptides generated by the β-amyloid precursor protein (βAPP)

and neurofibrillary tangles that consist of hyperphosphorylated tau protein (Rogaeva, 2002; Sherrington, 1995). According to Verheijen & Sleegers (2018) there are at least 25 genes associated with AD. Goate and her colleagues (1991) suggested that mutations in the APP gene could cause AD. The βAPP gene belongs to chromosome 21, which has been associated with Down's syndrome when there is an extra copy of chromosome 21 present. This extra copy of chromosome 21 generates harmful amyloid (National Institute on Aging). Thus, many people with Down's syndrome experience dementia and AD as they get older (Masters, 1985). Amyloid precursor protein can be processed by a couple of pathways, one of them is γ-secretase, which is important for the production of Aβ peptides (Rogaeva, 2002). It has been suggested that presenilins (PS1 and PS2) play role in the activity of γ-secretase (Rogaeva, 2002; Checler, 2001). Mutations on the gene PS1 in early-onset dementia is accounted for 18 to 50 percent. Apolipoprotein E (ApoE) gene, on the other hand, has been identified in late-onset AD patients (Sherrington, et al.,1995). Although Aβ plaques and tau tangles have been identified as the markers for AD, the main cause for cognitive impairment is the loss of synapses and neurons (Jellinger, 2006). It was confirmed by Goate et al. (1991) that AD pathology begins with β-amyloid peptide deposition. However, a very important research report by Riley, Snowdon & Markesbery (2002) complicated all the studies and knowledge about AD. By studying 130 nuns, they found many who had relatively small AD pathology but still developed clinical dementia, while others who had more severe AD pathology, showed little evidence of dementia. These results suggest that there are other factors that can trigger the development of the main symptoms of this neurodegenerative disease. One of these factors could be the inflammation in the brain or other lifestyle factors.

Two classic pathological hallmarks of the disease are Aβ plaques and tau tangles. Aβ plaques are formed when there is improper processing of a naturally existing APP. APP normally gets processed in its long form but in case of AD this improper processing leads to cleavage of its protein and formation of its small form (Aβ amyloid). These smaller forms aggregate together and create plaques. Tau tangles are formed when natural protein called Tau is hyperphosphorylated in the cells. Together Aβ plaque and tau are toxic to the surrounding cells and cause inflammation and cell death in the brain. Although this is the dominant theory of the pathology of the disease, some researchers believe that Aβ plaques are a defence mechanism that the body develops to fight and slow down the progression of the disease. Therefore, whether this is the cause, or the consequence of AD is still a controversy. However, in studies where Aβ plaques have been removed or reduced, animals have been performing better in cognitive and memory tasks.

#### 1.6.1.1.AMYLOID BETA PLAQUES (Aβ)

"Amyloid cascade hypothesis" is based on the idea that amyloid beta protein triggers events leading to AD pathology (Hardy & Higgins,1992) and production of amyloid β peptide is seen a decade before the clinical manifestations of AD. Aβ belongs to the "amyloid" (starch-like) group of proteins that forms insoluble extracellular deposits (Hardy & Higgins, 1992). Aβ is a peptide that derives from the amyloid precursor protein (APP), and creates amyloid plaques (Götz, Bodea & Goedert, 2018). It is suggested that increased levels of Aβ trigger neurofibrillary tangles and apoptosis (Hardy and Higgins,1992; Steen et al., 2005). This is supported by Akiyama et al. (2000) that Aβ accumulation causes inflammatory responses, which eventually leads to cell death.

Aβ can affect insulin signaling functions and inhibit insulin binding (de la Monte, 2009). Aβ peptide metabolism is influenced by insulin (de la Monte, 2009) which disrupts normal physiological processing. Although Aβ plaques have been an indicator of AD for more than hundred years, there is still uncertainty on how it is related to memory decline (Trinchese et al., 2004).

#### 1.6.1.2.NEUROFIBRILLARY TANGLES (TAU-PROTEIN)

It is not clear yet whether mutations in the tau gene are involved in AD. The hyperphosphorylation of tau could be based on the abnormalities in βAPP processing (Rogaeva, 2002; Hardy & Higgins, 1992); Aβ accumulation could promote tau hyperphosphorylation (de la Monte, 2009). Tau proteins that are associated with AD form neurofibrillary tangles (NFTs) that play role in cell loss and atrophy. Neurofibrillary tangles have been observed throughout the stages of the progression of AD (Jellinger, 2007), beginning in the (trans)entorhinal area (stages I and II) of the mediotemporal cortex, progressing to the limbic system (stages III and IV) involving hippocampus, amygdala, thalamus, hypothalamus and basal forebrain, finally, spreading to the entire neocortex (stages V and VI) (Riley, Snowdon, & Merkesbery, 2002). These disruptions cause impaired brain functions and dementia. According to Riley and colleagues (2002), it has been suggested that first two stages are the “silent stages”, and the limbic stages reflect clinical symptoms, when usually a patient or their family members notice problems.

Many studies suggest that tau phosphorylation is regulated by insulin and insulin growth factors (Schubert et al., 2004; de la Monte, 2014). Moreover, de la Monte et al. (2014) note that insulin and insulin growth factor signaling impairs tau gene

expression leading to AD pathology and neurodegeneration. Neurofibrillary tangles are composed of helical filaments formed from tau protein (Hardy & Higgins, 1992). Presence of NFTs disrupts the communication between axons (Gomez-Isla et al., 1997) and the number of tangles corresponds to the severity of the AD.

## 1.7.MOUSE MODELS OF ALZHEIMER'S DISEASE

Mouse models have been used extensively to study diseases; this is due to the genetic similarities of mice to humans, the well-developed methods for genetic manipulation, and the ability for mice to undergo complex behavioural testing in tasks that resemble those used to study human cognition. Studies on mouse models of AD help selectively target specific factors and treatments that are important in AD.

Why is there gene mutation modeling involved in rodents, such as mice? Simply stated, wild type rodents do not spontaneously develop Aβ plaques or NFTs (Götz, Bodea & Goedert, 2018). Mutations in genes that play a role in EOAD are used to create animal modeling for AD. These genes are APP, PSEN1 and PSEN2, also called PS1 and PS2. Both presenilin genes are participating in APP processing (Götz, Bodea & Goedert, 2018). Mouse models of AD are transgenically modified, meaning that its genome has been altered through genetic engineering to insert human genes that play a role in AD. Most of the genetically modified mice for AD hold mutations that are characterized in familial or early-onset AD (Götz, Bodea & Goedert, 2018). These transgenic mice models can express very similar neurohistopathologies to humans, Aβ plaques or NFTs. However, most models do not display both hallmarks and many transgenic mouse models do not show NFTs. Genetic modeling is not the only process for non-human animals to simulate AD. Non-genetic approaches also are available and have been used.

Homology-directed recombination (HDR) Alzheimer disease include both knockout (KO) and knock-in (KI) models (Götz, Bodea & Goedert, 2018). Knockout mice model means that a gene is inactivated while replaced with an implanted piece of DNA. Whereas, knock-in means that a specific gene is targeted and inserted into a specific locus area (Minikel, 2012).

Combinations of AD-related genes, such as in the APP/PS1 mouse model have been implemented in many research studies. These combinations are achieved through transgenic insertions of two or more mutant genes (Götz, Bodea & Goedert, 2018). There are many transgenic mouse models of AD, for example, there are double transgenic mouse, triple transgenic or more. For example, 5XFAD mouse model consists of APP and PS1 transgenes with five AD linked mutations (Alzforum.org).

No transgenic mouse model simulates the full phenotype of human AD, but they are still suitable for targeting therapeutic methods (Wong, Cai, Borchelt, & Price, 2002) and can provide important information about how factors may interact in this disease.

#### 1.7.1.1.APP/PS1 MOUSE MODEL OF ALZHEIMER'S DISEASE

The APP/PS1 mouse model is a double transgenic mouse that involves two proteins responsible for Aβ deposition in AD. The model includes both human transgenes (APP and PSEN1). This has been one of the most popular models used for AD research studies because of its rapid development of neuropathology. Both APP and PS1 are associated with early-onset AD. It is suggested that these mice develop Aβ plaques at six weeks in the neocortex, while plaque deposition in the hippocampus is found at three to four months (Alzforum.org). Other sources suggest that plaque deposition is observed at two months of age (Trinchese et al., 2004) and 6 to 7 months (Reiserer, Harrison,

Syverud, & McDonald, 2007). However, there are no neurofibrillary tangles observed in this model (Alzforum.org).

This mice model develops an impairment in long-term potentiation (LTP), which is important for forming new memories, as early as three months of age (Trinchese et al., 2004). In the study by Trinchese et al. (2004) they compared APP/PS1 mice of different ages with their wild type mates across multiple behavioural tasks. It was concluded that synaptic impairment is observed at late ages (five to six months), working memory is affected at three months, but reference memory is impaired after six months.

For the present study, APP/PS1 transgenic mice were used with expression of amyloid precursor protein (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9) (The Jackson Laboratory).

## CHAPTER 2: AD AND SUGAR

Based on the available literature and epidemiological studies, it is suggested that nutrition plays an important part in the development of AD. While there is no cure for AD and medication treatments are in the beginning stages, it is important to examine modifiable factors that affect the onset of AD. With increased use of Western diet and lack of exercise, people increase their chances of health problems such as obesity and impaired cognitive functioning. The Western diet consists of high dietary intake of sugar, saturated fats (animal products) and refined carbohydrate (with high glycemic index), and a relatively low intake of unrefined seeds (legumes), vegetables and sea food. (Kanoski & Davidson, 2011; Berrino, 2002). Saturated fats are a type of saturated fatty acids that contain predominantly animal fat products, and they have been shown in many studies that saturated fats impair cognitive function (Kanoski & Davidson, 2011).

The Western diet includes high calorie and high sugar intake which have been linked to risk of AD (Pasinetti & Eberstein, 2008). Unfortunately, while dietary sugar intake, such as fructose, has been increasing, the understanding of its effect on the brain may have been underestimated (Stranahan et al., 2008). Obesity in middle life is considered as a risk factor for dementia, and in the study by Pasinetti & Eberstein (2008) they concluded that obese participants with greater body mass index (BMI) have a 35 percent increased risk of getting dementia. In addition, obesity has been linked to chronic inflammation that can lead to insulin resistance and type 2 diabetes (Jimenez-Gomez et al., 2013). Meanwhile, the largest study ever done with two million people over two decades shows that being underweight in middle age and old age increases risk of dementia (Qizilbash et al., 2015). Possible reason that play some role in this observation could be the socioeconomic status. However, authors note that AD might show different associations with BMI. The obesity factor is complex and may not relate to sugar intake alone. It may also be true that high BMI in late life is protective against AD.

Nutrition is a fundamental factor affecting health and sugar can adversely affect health by causing inflammation in the body and in the brain. High sugar diets predispose to overweight and insulin resistance that can lead to type 2 diabetes and affect nutritional adequacy (Howard & Wylie-Rosett, 2002). Experimental studies found that exposure to nitrosamines that can be found in processed and preserved foods, cause cognitive impairment, insulin resistance in the brain, and AD-like neurodegeneration (de la Monte, 2014).

A high sugar diet and high fat diet cause obesity and inflammation in the body and in the brain according to Kim & Feldman (2015), while inflammation causes increased

Aβ deposition. When glucose is not metabolised in the tissues, insulin resistance can lead to tau protein pathophysiology and Aβ deposition (Kim & Feldman, 2015). These pathological accumulations lead to disrupted neuronal connections and neurodegeneration.

Cao et al. (2007) demonstrated in a transgenic mouse model of AD that sucrose-sweetened water resulted in insulin resistance and impaired memory performance on behavioural tasks, as well as increased Aβ deposition. Meanwhile, Murphy and Johnson (2003) argue that high sugar diets are associated with many health problems. According to Stranahan et al. (2008) rats were fed with high fat, high sugar diet for eight months and found that it impaired their hippocampus-dependant learning, reduced levels of BDNF in the hippocampus, synaptic plasticity, and dendritic spine density.

When progression of AD worldwide is compared to progression of processed food and sugar consumption, the link between both is notable (Shahbandeh, 2019). Given the variety of readily available sources of large amounts of sugar in many foods, this statistic is not surprising. According to the World Health Organization, the recommended daily intake of sugar is 25 grams. Based on data from 2015, average Canadian children aged two to eight consume 101 grams of sugar a day, children aged nine to 18 consume 115 g/day, while adults consume 85 g. This statistic shows reduction of sugar intake from data collected in 2004, which stated that the average Canadian consumes 110 grams of sugar a day (Canadian Sugar Institute, 2015). Review by Langlois & Garriguet (2011) found that lowest sugar consumption was among women aged 71 and older (83 grams or 20 teaspoons), with highest among teenage boys aged 14 to 18 years old (172 grams or 41 teaspoons). Langlois & Garriguet (2011) found that across all age groups, males

consumed significantly more sugar. However, studies show that females have higher risk of getting dementia and/or AD (Qizilbash et al., 2015). This confirms the complexity of AD and sugar interaction. In the study by Murphy & Jonson (2003) almost half (44%) of the average daily sugar intake was from beverages, such as milk, fruit drinks and soft drinks. Meanwhile, the average amount of consumed added sugars in diet was increasing (Murphy & Jonson, 2003). One Tim Horton's medium size hot chocolate drink consists of 49 g of sugar, while a chocolate chip muffin has 36 grams (Fatsecret.ca). This statistic alone, creates concern on how our health is affected by processed and sweetened foods. As statistical review by Langlois and Garriguet shows that most of the dietary sugar that is consumed comes from added sugar in beverages, salad dressings, syrup and candy, besides natural sugar that is found in fruits, dairy and vegetables. Studies show choosing added sugar instead of natural sugar increases micronutrient deficiency (Murphy & Johnson, 2003) because the sources of natural sugars have many components necessary for healthy metabolism, such as antioxidants (Phillips, Carlsen, & Blomhoff, 2009). Added sugars should be avoided because of their lack of additional nutritional value and the fact that fat-free processed foods are often high in calories due to high amounts of sugar (Howard & Wylie-Rosett, 2002). It should be a valuable choice to consider quantity and quality when choosing carbohydrate foods, including sugar (Pasinetti & Eberstein, 2008). When people are controlling carbohydrate foods, better insulin sensitivity, glycemic control and need for fewer medications has been observed (Volek & Feinman, 2005). If nutrition and exercise are factors that can prevent or delay from developing this neurodegenerative disease, then a healthy lifestyle should be a sense of urgency if not common sense.

## 2.1.INSULIN RESISTANCE

Insulin is a hormone that controls the balance of sugar levels in the body and has activity through the hypothalamus to regulate metabolism and food intake. Insulin is also associated with cognitive functions such as memory within hippocampus (McNay et al., 2010) and neural development (Liu, Liu, Grundke-Iqbal, Iqbal, & Gong, 2011). It regulates multiple processes within the brain, such as homeostasis, reproduction, sympathetic activity, neuronal proliferation, apoptosis, synaptic transmission, learning and memory (Rojas & Gomes, 2013; Gupta et al., 2011). Insulin is released by the pancreas when the food is consumed to use and store glucose for energy. Meanwhile, insulin in the brain is produced by the pancreatic β-cells and pyramidal neurons in the hippocampus, prefrontal cortex, entorhinal cortex, and the olfactory bulb (Hoyer, 2002). Insulin resistance is defined by impaired responsiveness to normal levels of insulin and is a major marker for type 2 diabetes. By accelerating APP, insulin may significantly increase Aβ accumulation (Pasinetti & Eberstein, 2008). Diabetes mellitus is caused by disrupted insulin signaling (de la Monte & Wands, 2008) and this disruption is also observed in neurodegenerative disorders (Schubert et al., 2004) such as AD patients (Kim & Feldman, 2015). Type 1 diabetes mellitus (T1DM) is an autoimmune response to the organism that attacks pancreas and blocks insulin production. Type 2 diabetes mellitus (T2DM) is associated with insulin resistance due to lifestyle factors, such as nutrition, lack of exercise and inheritance.

Glucose uptake in the brain is regulated by insulin that controls expression of glucose transporter (GLUT) proteins (de la Monte, 2014). Insulin and insulin receptors (IR) are located in the central nervous system (CNS), while the largest expression of IRs

remain in the hypothalamus (Schubert et al., 2004; Moroo et al., 1994). McNay et al. (2010) state that insulin regulates food intake and metabolism via the hypothalamus. Insulin may also act on the hippocampus, which is known to possess high levels of insulin receptors, suggesting its importance in synaptic plasticity and memory (McNay et al., 2010; Izumi, et al., 2003). McNay et al. (2010) wanted to determine if insulin delivered to the hippocampus could modulate memory processes. In this study, they showed that local delivery of insulin to a rat's hippocampus enhanced spatial memory and glycolytic metabolism, while the delivery of insulin outside of hippocampus did not have an effect on memory performance. They concluded that insulin is critical for hippocampal memory processing.

It is argued that insulin signaling, and insulin-like growth factors, have associated roles in the pathogenesis of AD, affecting Aβ production in the brain (Kim& Feldman, 2015; de la Monte & Wands, 2008). Disruption to insulin production and signaling affects memory and cognition, thus insulin resistance in the brain may play a role in cognitive impairment observed in AD patients (Frölich et al., 1998; Gupta, Bisht & Dey, 2011). Moroo et al. (1994) supports this theory by stating that reduced expression of IR is common among AD and Parkinson's disease patients. These insulin metabolism abnormalities influence the synthesis and degradation of Aβ peptides (Pasinetti & Eberstein, 2008).

Kim and Feldman (2015) reports that insulin travels to the brain through the blood-brain barrier and insulin resistance in the brain plays a major role in cognitive impairment and development of AD, as shown in both human and nonhuman animal studies (Kim & Feldman, 2015). Kim and Feldman (2015) also note that the hippocampus

and temporal lobe are brain areas that are early targets for AD development and these locations have shown the highest insulin resistance density. According to Fink, Kolterman, Griffin & Olefsky (1983) as people age, insulin sensitivity declines, even with no clinical symptoms of T2DM. Insulin resistance is associated with cognitive deficits observed in T2DM and dementia, which include AD (McNay et al., 2010). Similarly, Talbot et al., (2012) showed that insulin resistance increased from cognitively normal to AD subjects with higher density in amyloid plaque deposition. This was supported by Liu et al. (2011) in comparing AD and T2DM post-mortem brains to controls and discovering that insulin signalling pathways were decreased in diseased brains. Willete et al. (2015) found that Aβ deposition was increased in subjects with higher insulin resistance levels. De la Monte & Wands (2005) support the idea that Aβ cause neurotoxic cell death, and also impairs insulin binding and insulin phosphorylation. Hoyer (2002) suggests that abnormal insulin and IR expression in the brain leads to reduced levels of acetylcholine and decreased levels in cerebral blood flow. Multiple studies strongly suggest that impaired insulin receptor signaling that causes insulin resistance is the culprit for AD pathology, thus causing elevated γ-secretase activities (Pasinetti & Eberstein, 2008).

Glucagon-like peptide-1 (GLP-1) is a hormone that utilizes glucose levels in the body's tissues and nervous system, more importantly it can cross the BBB and enhance insulin signaling. GLP-1 is an important hormone that can promote synaptic plasticity, cell survival and cognition (Kim & Feldman, 2015). Since, GLP-1 is an important component for insulin signaling, its impairments may be related to diabetes and cognitive function deficits. While most of the studies conclude that T2DM is associated with impaired cognitive function and risk factor for AD, Jolivalt et al. (2008) states that in

mouse models of T1DM there is reduced insulin signaling and hyperphosphorylation of tau and amyloid beta similar to AD brains.

## 2.2.INSULIN-LIKE GROWTH FACTORS (IGFs)

Insulin and insulin-like growth factors (IGFs) are important hormones for metabolic and developmental processes that control survival of neurons and plasticity (de la Monte, 2009). It also contributes to neuronal growth, migration, metabolism, gene expression and synapse formation (D'Ercole, Ye, Calikoglu, & Gutierrez-Ospina, 1996). Therefore, IGF signaling pathways are crucial for cognitive function (de la Monte, 2014). Insulin and IGFs are expressed in the brain and if these peptides are reduced, it is associated with AD (Steen et al., 2005). Impaired growth factor signaling produces growth factor withdrawal, which leads to neuronal death (de la Monte & Wands, 2008), which is exactly what happens in AD mechanisms. In other words, impaired signaling that is caused by reduced levels of growth factor expression initiates insulin and IGF-I resistance in the brain (Steen et al., 2005). There are two peptides – insulin-like growth factor 1 (IGF-I) and insulin-like growth factor 2 (IGF-II) – that play an important part in insulin mechanisms. According to D'Ercole et al. (1996), IGF-I influence development of most brain regions with cerebellum and cerebral cortex being the most sensitive. It has been observed in research studies that abnormal mechanisms in insulin and IGF signaling produces increased oxidative stress, mitochondrial dysfunction, proinflammatory cytokine activation and APP expression (de la Monte & Wands, 2008; de la Monte & Wands, 2005). Similarly, reduced expression of neuronal genes and inflammatory mechanisms of AD contributed to IGF abnormalities and insulin resistance in the brain (de la Monte & Wands, 2008).

Steen et al. (2005) demonstrate that impaired levels of IGFs are observed in AD brains. Their study looked at 54 post-mortem brain tissues comparing healthy and AD samples and observed that the highest growth factor expression was in hippocampus and hypothalamus, with insulin gene expression highest in the hippocampus in controls. While in AD brains there is a reduced insulin and IGF-II gene expression in hippocampus and hypothalamus, and reduced levels of IGF-I gene expression in the frontal cortex and hypothalamus. These findings make sense if we consider that AD expresses first deficits in memory impairment and may be related to the insulin and IGFs expression. They found that reduced insulin gene expression is found in AD cerebral tissue, suggesting that insulin deficiency occurs globally around the brain.

## 2.3.TYPE 3 DIABETES

AD is frequently popularized as type 3 diabetes, because of the insulin resistance developed in the brain (Kim & Feldman, 2015; Steen et al., 2005). This is due to the suggestions of insulin deficiency and insulin resistance being factors for AD-type neurodegeneration observed in type 2 diabetes mellitus (T2DM) (de la Monte & Wands, 2008; de la Monte, 2009). De la Monte (2009) argues that insulin resistance or reduced insulin actions in the brain cause AD-associated abnormalities in energy metabolism, in other words, brain diabetes. Due to the features of type 1 (insulin deficiency) diabetes and type 2 (insulin resistance) that are common in AD, it is proposed as “type 3 diabetes” (de la Monte, 2014). While type 1 diabetes mellitus (T1DM) is expressed due to genetic factors, T2DM is caused by lifestyle factors. Both diseases are life threatening because the body does not produce enough insulin to control glucose levels. T2DM is associated with impaired cognitive functions, including memory, due to hyperglycemia and insulin

resistance (McNay et al., 2010). As Leibson et al. (1997) noted, many diabetic comorbidities, such as stroke, hypertension, depression, have been associated as risk factors for development of dementia and AD.

Steen et al. (2005) emphasize that AD pathology is related not only to the insulin resistance but also to the growth factor gene expression. It is suspected that T2DM might not be a mechanism to cause AD but can influence the progression and pathogenesis of the disease (de la Monte & Wands, 2008). According to a study by de la Monte and Wands (2005), insulin resistance could play a role in AD neurodegeneration processes by impairing cerebral microvascular and choroid plexus functions, leading to hypoperfusion and compromise of the blood-brain and blood-CSF barriers. When looking at human post-mortem brains, researchers found that advanced AD patients had reduced levels of insulin and IGF-I peptides in the brain, which starts with insulin and IGF signaling abnormalities in early AD stages and worsens with the progression (de la Monte & Wands, 2008). They also discovered abnormalities in AD human brains that were similar to those observed in T1DM and T2DM. Such abnormalities included IGFs, therefore, it was suggested to be referred as "type 3 diabetes" (de la Monte & Wands, 2008). Post-mortem AD lesion studies show that IGF and insulin signaling mechanisms are destroyed (de la Monte & Wands, 2008). Meanwhile, animal studies support that mice with deficient insulin receptor substrate or neuronal insulin receptor gene express reduced brain growth and increased *tau* phosphorylation (Steen et al., 2005).

Cognitive impairment is observed in diabetic elderly, especially involving recent memory tasks and executive functioning, as reported by Verdelho et al., (2007). It has been suggested that diabetes and hypertension (Launer et al., 2000) are the greatest risk

factors for cognitive decline and dementia (Verdelho et al., 2007; Arvanitakis, Wilson, Bienias, Evans, & Bennett, 2004; Ott et al., 1999). In fact, Launer et al. (2000) suggests that elevated blood pressure in mid-life adulthood is linked to AD. 80 percent of AD patients have also T2DM or insulin resistance (Kim & Feldman, 2015) and according to Leibson et al. (1997) 65 percent of T2DM patients have increased risk to develop AD. In contrast, MacKnight, Rockwood, Awalt, & McDowell (2002) argue that there is no association between AD and diabetes, although they found that there is association between diabetes and vascular cognitive impairment. According to a longitudinal study by Arvanitakis et al., (2004) where nuns, brothers and priests of age 55 or older were examined, those with diabetes mellitus had 65 percent increased risk of developing AD.

According to de la Monte (2014) the strongest evidence for concept AD as type 3 diabetes is based on the rodent studies. For example, rats were administered intracerebroventricular injections of streptozotocin, which induced diabetes. These rats developed cognitive impairment, spatial learning and memory deficits, as well as insulin resistance and insulin deficiency in the brain. In addition, AD-like pathology and neurodegeneration was observed.

Insulin, IGF-I and IGF-II are located in the brain; thus AD is associated with reduced levels of growth factor gene expression in the brain (Steen et al., 2005). Insulin and IGF signaling abnormalities in the brain, express different processes than T1DM and T2DM, therefore “Type 3 diabetes” is the term that corresponds to brain insulin resistance associated with AD. Nevertheless, AD and T2DM prevalence increases with age and both diseases have genetic components (Janson et al., 2004).

# CHAPTER 3: AD AND METFORMIN

Due to the overlapping molecular and pathological similarities between diabetes and AD, many anti-diabetic drugs have been evaluated for their effects on AD. Since inflammation in the brain is associated with the AD and Aβ accumulation, treatments that focus on anti-inflammatory properties have been a focus point (Meraz-Ríos et al., 2013). One of these is metformin, an anti-diabetic drug, that has been used widely in T2DM patients and which has anti-inflammatory properties (Markowicz-Piasecka, Sikora, Szydłowska, Skupień, Mikiciuk-Olasik, & Huttunen, 2017).

Since insulin resistance and abnormal insulin signaling is associated with Aβ deposition (Kim & Feldman, 2015), multiple anti-diabetic treatments are being tested in regard to possible therapeutic processes for AD. Several treatments with insulin sensitizers have been used due to the theory of impaired insulin signaling playing a role in AD pathogenesis (Liu et al., 2011). Metformin reduces glucose levels in the blood and controls insulin. It has been used to treat people with diabetes while showing properties of improving cognition. Gupta et al. (2011) demonstrated that metformin improved impaired insulin actions and prevented from AD-like pathologies. Importantly, Wang et al. (2012) demonstrated in cultured human and rodent tissues processes of neurogenesis.

While metformin is known for its therapeutic properties as an anti-diabetic agent, there are several studies that tested metformin effect on other neurodegenerative diseases and cancer as a potential treatment (Rojas & Gomes, 2013). In the study by Ma et al. (2007) metformin therapy was used among transgenic mouse models of Huntington's disease. They found that metformin increased longevity in mice and improved clasping motor defect. Studies that have been used to test metformin have shown improvements in

both humans and nonhuman animals (Reger, et al., 2008). Several studies demonstrated that cancer incidence rates were decreased, as well as cancer-related mortality rates with the treatment of metformin (Rojas & Gomes, 2013; Saisho, 2015). In addition, metformin may improve the efficacy of overcoming the chemotherapy resistance (Markowicz-Piasecka et al., 2017). Although metformin is shown to reduce cancer cell growth, the interaction between both cannot be explained yet (Rojas & Gomes, 2013).

### 3.1. METFORMIN AND TYPE 2 DIABETES

Metformin's main effect is control of hyperglycemia, but it has many others, such as improvements in endothelial dysfunction, homeostasis and oxidative stress, insulin resistance, lipid profiles, and fat distribution (Rojas & Gomes, 2013). In addition, studies that compared metformin to other anti-diabetic drugs among diabetic patients, showed improved cognitive function in those who received metformin treatment (Markowicz-Piasecka et al., 2017).

T2DM has dramatically increased among the pediatric population. In fact, between 1990 to 1999 incidence reports increased from 8% to 45% depending on the geographical location (Rojas & Gomes, 2013). Considering the variety of processed and highly sweetened foods that are available to children today, this incidence report most likely has doubled by now. Metformin has also been used by children and adolescents showing beneficial results (Rojas & Gomes, 2013).

Some of the side-effects associated with metformin use, are gastrointestinal intolerance, vitamin B12 deficiency and the more serious, but rare, lactic acidosis, which results from severe tissue hypoperfusion (Rojas & Gomes, 2013; Maruthur et al., 2016). On the other side, studies have demonstrated that metformin decreased production of β-

amyloid in cell culture models and *in vivo,* as well as, ameliorated oxidative damage (Markowicz-Piasecka et al., 2017). Oxidative stress is observed among patients with diabetes, therefore, Mirmiranpour et al. (2013) compared lifestyle modifications with treatment of metformin or pioglitazone among T2DM patients. They found that both anti-diabetic medications had beneficial effects on the markers of oxidative stress.

In the study by Guo et al. (2014) 58 patients diagnosed with T2DM and depression were recruited; half were treated with metformin and half with a placebo for 24 weeks. At the end of treatment, subjects were tested on memory tests and depression recording scales. The results indicated that the metformin group, compared to placebo, showed significantly enhanced verbal memory scores, visual memory scores, attention and concentration and delayed memory scores measured with the Wechsler Memory Scale Revised (WMS-R). As noted by Guo et al. (2014) cognitive function is related to depression, therefore, subjects were given pre- and post-tests for depression in which the group that was treated with metformin showed significantly decreased scores on Montgomery-Asberg Depression Rating Scale (MADRS) and Hamilton Rating Scale for Depression (HRSD-17). These results indicate that metformin improved cognitive performance and depressive symptoms among subjects. In this study, metformin also reduced HbA1c plasma levels related to blood sugar levels, which means that diabetes was controlled well.

### 3.2.AD AND METFORMIN: ANIMAL STUDIES

As mentioned above, metformin plays a role in modulating cognitive functioning, including learning and memory. In the study by Ashrostaghi, Ganji, & Sepehri (2015), researchers demonstrated that metformin administration in 24-months-old rats improved

performance on the Morris water task, which tests spatial memory. Allard et al. (2016) conducted a study in which 48 C57B1/6 mice were assigned to either a regular rodent diet, high fat diet or high fat diet plus metformin. The results demonstrated that metformin reduced body fat, and improved motor coordination. In addition, Oliveira et al. (2016) found that metformin improved spatial memory performance in mice, while also decreased neuroinflammation and loss of neurons in the hippocampus. Wang et al. (2012) demonstrated that metformin increased hippocampal neurogenesis and spatial memory in mice, while Allard et al. (2016) showed increased neural growth and synaptogenesis in mice.

There are studies that demonstrate that metformin increases AD-like pathology. For example, Picone et al. (2015) treated C57B6/J(B6) four weeks young mice with metformin and showed that there was an increased Aβ aggregation, as well as aggregated APP and PS1 gene expression. It is important to mention that this treatment lasted for seven days.

### 3.3.AD AND METFORMIN: HUMAN STUDIES

While the interaction between metformin and AD is not fully understood, the suggestion is that certain pathways regulating neural stem cells might be responsible for the neuroprotective properties (Markowicz-Piasecka et al., 2017). According to Markowicz-Piasecka et al. (2017) metformin decreases the activity of acetylcholinesterase (AChE), which breaks down acetylcholine (Ach), a neurotransmitter involved in learning and memory among other things.

While there are several studies that show beneficial properties of metformin among AD patients, there are some studies also among humans that suggest metformin

can worsen AD. Such a study was designed by Imfeld, et al. (2012) in which they recruited over 7,000 AD patients with diabetes with a matched number of controls between 1998 and 2008. They found that long-term users of metformin were at greater risk of developing AD. Despite the study by Imfed et al. (2012), many studies, reviewed by Markowicz et al. (2017), conclude that metformin has been identified with anti-cancer and anti-aging properties. In addition, metformin has been shown to ameliorate oxidative damage *in vitro* and *in vivo* studies (Markowicz-Piasecka et al., 2017). Most importantly, it has been recognized as a potential benefit in people with AD.

# CHAPTER 4: THE PRESENT STUDY: EFFECT OF HIGH DIETARY SUGAR AND METFORMIN IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

## 4.1. INTRODUCTION

Alzheimer disease is responsible for 80% of all dementia cases (Harvey et al., 2003; Verheijen & Sleegers, 2018) and has been a devastating disease starting with neurotoxic changes in the brain that lead to brain shrinkage and eventual death. According to several studies, Type 2 diabetes is associated with higher risk of developing Alzheimer disease (Cao, Lu, Lewis & Li, 2007; Markowicz-Piasecka et al., 2017). In fact, Zhang et al. (2017) published a meta-analysis of 17 studies which involved 1,746,777 subjects and concluded that patients with diabetes had significantly higher incidence of AD. In addition, Chen and Zhang (2013) found that diminished cerebral glucose metabolism lead to impaired glucose homeostasis and neuronal impairment. Type 3 diabetes has been popularized due to the similar characteristics of diabetes and dementia such as impaired insulin actions and insulin resistance in the brain, as well as cognitive

impairment. According to de la Monte (2014) glucose is the main energy source for the brain, therefore, impairments in glucose uptake can lead to "starvation" of the brain. This starvation of the brain comes with oxidative stress, impaired homeostasis, and neuronal death (de la Monte, 2014). While the relationship between diabetes and impaired cognition is close, it is fair to admit that there are multiple co-factors, in other words - risk factors, that lead to AD. Multiple co-factor theory states that genetics, lifestyle factors, head injuries, sleep disturbances, seizures, and neuronal changes, to name a few, could play a role in development of AD (McDonald et al., 2010). Meanwhile, Markowicz-Piasecka et al. (2017) continues with a list of changes that have been associated with diabetes and cognitive impairment. These changes are macro and micro-vascular - hyperinsulinemia, insulin resistance, and chronic inflammation. All these changes can lead to disturbed neuronal function and cell death, processes seen in AD brains.

Considering the common pathologies between AD and diabetes, therapeutics used for diabetic patients could be effective for AD. One of these pharmaceutical therapies is metformin (Chen & Zhong, 2013). Metformin is an anti-diabetic drug that increases glucose uptake in muscles (Saisho, 2015) and has many beneficial properties observed in rodents and humans. Type 2 diabetes is due to insulin resistance and sensitivity which can be a result of excessive sugar consumption. Research studies with transgenic mouse models of AD have shown that high-fat diets increase AD-pathology and cognitive impairment (Cao et al., 2007, Abbott, Morris & Reichelt, 2016). In addition, Abbott et al., (2016) argues that dietary sugars impair hippocampal-dependent memory consolidation and retrieval, reduces hippocampal dendritic spine density, long term potentiation, neurogenesis and increases levels of pro-inflammatory markers. Thus, in the present

study it was suggested that a high-sugar diet could contribute to AD progression. To further understand the relation between AD and high sugar, meanwhile, testing the effect of metformin on AD, we studied the effect of sugar and sugar-free chocolate puddings and metformin on APP/PS1 mouse model of AD, that expresses the human genes mutations. To measure the effect of treatment on AD mice, behavioural tests and histology assessments were performed. Measures on the balance-beam test were walking score, foot slips and falls; on the novel object recognition test, measures were time spent observing novel object and old object, discrimination index, investigation ratio of probability distribution, stops made and distance traveled. In the context fear conditioning test measures taken were stops made, distance traveled, freezing time and speed. Considering the fact that highest added sugar intake is among adolescents and young adults (Bremer & Lustig, 2012), two-month old APP/PS1 mice were subjects to the beginning of the study. It was hypothesized that high sugar intake may aggravate AD pathology, while metformin could ameliorate AD.

## 4.2.MATERIAL AND METHODS

### 4.2.1. ANIMALS

APP/PS1 (strain name: APPswe,PSEN1dE9)85Dbo) (N=32) of both sexes (16 females, 16 males) were obtained from the Jackson Laboratory, Maine. These mice express mouse/human amyloid β precursor protein containing K595N/M596L Swedish mutations and a mutant human presenilin 1 carrying the exon 9-deleted variant, directing the transgene expression to the brain (Cao et al., 2007). After arrival from the JAX laboratory, animals were acclimated to the vivarium for a week. Then animals were placed in a reverse dark cycle, from noon to midnight in the light phase. Animals were

single housed or up to four per cage with free access to regular chow food pellets and water with daily two-hour restricted access to food during treatment period. Animals were almost ten weeks-young at the beginning of the treatment. Treatment consisted of oral administration of either sugar free or regular chocolate pudding and metformin was mixed with either pudding according to the group for eight weeks. All work was in compliance with the Canadian Council of Animal Care and the regulations of the Province of Alberta and the University of Lethbridge Animal Welfare Committee.

### 4.2.2. EXPERIMENTAL DESIGN

Before the beginning of the treatment, animals were randomly assigned into four groups with eight mice of equal number of sexes in each group (n=8/group). The control group had only six mice in total (three females, three males) because one mouse had to be euthanized upon arrival from the JAX laboratory and another mouse died during the experiment from unknown reasons. Therefore, originally there were 32 mice ordered, but 30 were used for data collection and reports. All mice were maintained on a 12hr light/12 hr dark cycle in room temperature at 22 °C. These four groups of mice received treatment accordingly: sugar, sugar with metformin, sugar free with metformin and just sugar free (control).

In the beginning of treatment, mice were habituated to the sugar-free chocolate pudding for four days. Treatments were given every day two hours before the light cycle begins. This decision was made due to the fact that most of the caloric intake (80%) in mice occurs during the dark cycle (Kentish, Vincent, Kennaway, Wittert & Page, 2016). Therefore, to make sure mice consume their treatment, they were taken out of their home cages every day at 10 am and put in single temporary cages for the dose control purposes.

This way, each mouse received their own plate with chocolate pudding and metformin based on their weight, while given enough time to orally consume it. In fact, all mice in each group consumed both sugar-free and regular chocolate puddings without hesitation, approximately within five minutes. Once the lights were on at noon, each mouse was returned to their home cages with other mates, if applicable. Body weight was measured every other day.

After eight weeks of respective diets, mice were tested in a battery of behavioural tests followed by assessment of AD-neuropathology with histology procedures (described below). Behavioural procedures were conducted during the light cycle by testing their learning and memory, balance and motor coordination. At the end of the experiment, mice were anesthetized with isoflurane and perfused.

### 4.2.3. METFORMIN AND DIETS

Mice were assigned randomly into four groups with both sexes in each group. In addition to the regular rodent diet, as a treatment, mice received either full sugar or sugar-free chocolate puddings with metformin added in two groups. One group received just chocolate pudding (sugar), second group received chocolate pudding mixed with metformin (sugar + metformin), third group received sugar-free chocolate pudding mixed with metformin (no sugar with metformin) and a fourth group received only sugar-free chocolate pudding (control group). Each mouse consumed daily treatment of 0.7 g of pudding orally, with metformin dose (10mg/kg) given based on their weight, accordingly. This choice was made comparing other experimental studies, such as Kim et al. (2015). Metformin stock solution (50mg/ml) was prepared approximately every 10 days and kept in the -20$^{oC}$ freezer. Daily working concentration was three mg/ml (60 µl of stock

solution + 940 µl of $dH_2O$). Daily dose for each mouse was calculated based on their weight. For example, a mouse that weights 21 mg received 70µl of metformin daily working concentration (0.021 kg x 10 mg/kg = 0.21 mg / 3mg/ml = 0.070 → 70µl).

## 4.3. BEHAVIOURAL ASSESSMENTS

A battery of standard tests was used to assess behavioural performance on three tasks, novel object recognition task (NOR), balance-beam task (BBT), and context fear conditioning task (CFC). All behavioural tests were conducted right after the eight-week treatment period. These tasks are commonly used to study Alzheimer's disease mice because AD pathology leads to progressive decline in several forms of memory and motor function (Antunes & Biala, 2012).

### 4.3.1. NOVEL OBJECT RECOGNITION TEST

Novel object recognition test is used to assess visual recognition memory and investigation into memory alterations (Grayson, et al., 2014). Meanwhile, it can measure short-term memory, attention, anxiety, and preference for novelty (Antunes & Biala, 2012). This task is easy to conduct because it does not require external motivation, reward or punishment. It is based on the idea that mice will explore novel items based on their natural propensity to explore novel object more if their cognition is not impaired (Antunes & Biala, 2012). When mice are trained to explore identical objects, they will spend more time exploring the novel object (Mehla et al., 2019). This task is sensitive to parahippocampal regions of the temporal lobes (Antunes & Biala, 2012), therefore, if the performance is impaired there might be damage to these brain areas. In the current study, the test took three days; on the first day mice were habituated to the environment and

given eight minutes for exploration in the open-field arena in the absence of objects. The open field arena is white square testing box (47cm x 50cm x 30cm). 24 hours after habituation day, on the second day (familiarization) (Fig.1), mice were placed in the same environment with two identical objects (A + A), while given eight minutes to explore. On the third day (test phase) (Fig.1) after 24 hours of the familiarization day, mice were placed in the same environment with one object previously shown and one novel object (A + B) (Antunes & Biala, 2012) while for half of the mice, the novel object was placed on the right side and other half on the left side opposite corner. The preference for a novel object means that an animal remembers previously explored identical objects. Therefore, the object recognition in animals is measured by the difference in time spent exploring the novel and familiar objects. Between each mouse, objects and the box were cleaned with 1% Virkon to reduce any odor left from the previous mouse. A video camera was set up to record test phase performance for further analysis. The exploration at novel object and old object was measured by automated Matlab program tracking center of the body with approximate distance of 5 cm to the object and or touching the object with the nose (Antunes & Biala, 2012). The discrimination index (DI) was calculated by using the formula (time spent with novel object- time spent with old object)/total time spent with both novel and old objects). Investigation ratio measurement shows the mean probability of mice investigating the novel object vs old. The average number of stops and distance traveled between these stops was another measurement that could lead to approximate anxiety indication.

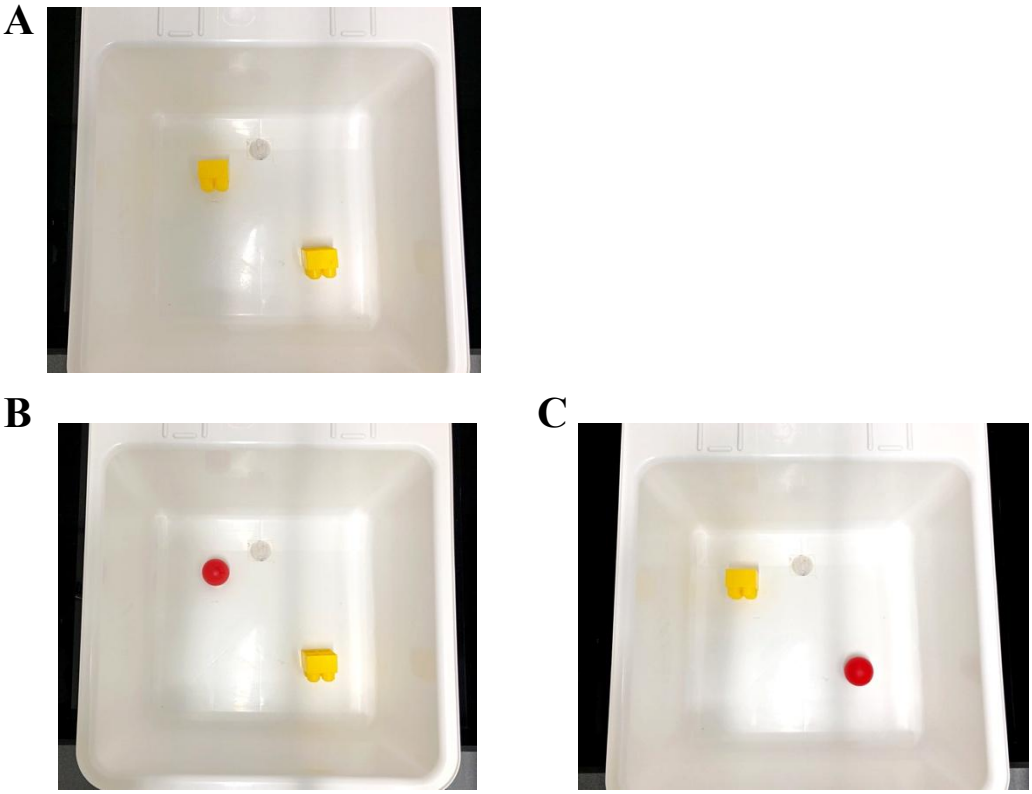



Figure 1. Novel Object recognition Test. (A) Familiarization phase. Animals explore two identical objects, A+A. (B) Test phase. Half of animals explore one previously shown object and one novel object, B+A. (C) Test phase. Other half of animals explore one previously shown object and one novel object, A+B.

### 4.3.2. BALANCE – BEAM TEST

Balance-beam test is used to assess balance and motor coordination. This test consists of a round beam (1 cm diameter, 100 cm long) that is elevated 50 cm above the ground with a foam pad cushion underneath the beam (Fig.2), in case the mice fall. Mice were brought to the testing room where the apparatus was prepared, and each mouse was placed on the beam one by one. Animals were encouraged to cross the beam and reach the other end of it by entering black cylinder at the end of the beam, which is the goal for the task. Mice were given three successful practice trials on the training day to cross

the elevated round beam. Then on the second day, the test phase is performed with three successful trials per mouse. The purpose of the training day is to reduce natural aversion to cross unprotected places. Measurements are taken to analyze number of times their hind-feet slip from the beam (Tung, Burton, Quail, Mathews & Camp, 2016), number of times they fell off the beam, as well as walking score, which results in scoring for their foot placement on the beam. These measurements target motor cortex.

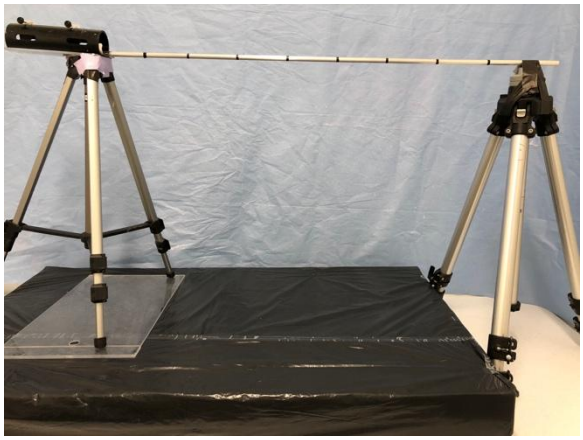


Figure 2. Balance-Beam Test. This test consists of a round beam (1 cm diameter, 100 cm long) that is elevated 50 cm above the ground with a foam pad cushion underneath the beam.

### 4.3.3. CONTEXT FEAR CONDITIONING

This test is used to assess amygdala and hippocampus dependent memory (Mehla et al., 2019). This test is useful to identify associative memory. In other words, if a mouse does not differ in behaviour in the novel context, where it was not shocked, then it cannot dissociate two environments. The test takes place in two rooms; one is for shock paired context and the other is for novel environment. The shock context is an acrylic square box (33x33x25cm) and the floor of this chamber is connected to 64 stainless steel rods (2mm diameter) 5 mm apart (Fig.3). The chamber is connected to the power generator for

delivery of a mild shock exposure for two seconds. Between each rodent's test, the chamber was cleaned with 1% Virkon. The novel context (triangle box) (Fig.3), novel room was used for measurement of freezing in a new environment, while freezing was also measured in a paired context phase in which animals were placed in the same shock box but without actual shock provided. Each of the context phases were recorder to measure freezing time (immobility), stops made and distance traveled, as well as the speed with which mice traveled. On the shock day, mice are put in the chamber for two minutes to explore and habituate to the environment. Then a two second, 0.5 mA shock was produced for five times with two-minute intervals in between. After the last shock, the mouse is left in the chamber for one minute. During the same context (A) and novel context (B) phases, mice are exploring the environment for five minutes. This behavioural test is performed as the last one due to the elevated stress and anxiety.

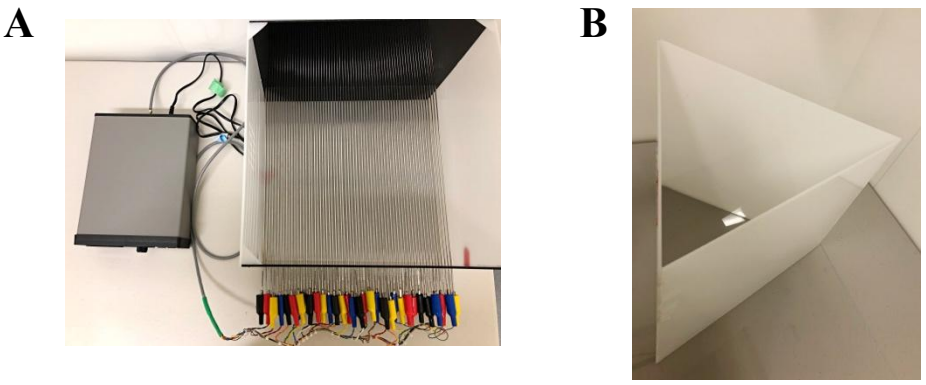



Figure 3. Context Fear Conditioning. (A) shock context chamber that is connected to 64 stainless steel rods (2mm diameter) with 5 mm apart. Chamber is connected to the power generator that provides mild shock. (B) Novel context, which is located in a different environment and shaped as a triangle.

### 4.4.HISTOLOGY

Mice were euthanized with intraperitoneal injection overdose of sodium pentobarbital. Once the death was assured, the heart was exposed by cutting through the abdomen and the rib cage. A small incision cut in the right atrium was done, while (~50 ml) 0.9 phosphate buffered saline (PBS) was pumping into the left ventricle. This was followed by (~50 ml) of 4% paraformaldehyde (PFA), until the blood turned to nearly clear. Once completed, the head was detached from the body and the brain carefully extracted. Brains were fixed in 4% PFA for 24 hours followed by immersion in 30% sucrose for at least 48 hours before being sectioned coronally at 40 μm thickness with a sliding microtome. 1/6 series were mounted onto superfrost+ slides and processed for immunohistochemistry to quantify Aβ plaques and microglia intensity, which corresponds to the inflammation.

Slides were fixed in 4% PFA for 4min, washed in TBS (Tris buffered saline) then placed in 70% formic acid for antigen retieval (5.5-8min). After rinses in TBS slides were permeabilised in TBS with 0.1% Triton-X for 15 minutes then blocked in TBS with 0.1% Triton-X and 2% BSA (bovine serum albumin) for 30 minutes. Primary antibodies 82E1 (anti- β-amyloid (N), IBL, 10323, mouse) and Iba1 (Rabbit, SAF4318, 019-19741, Wako) were added at a concentration of 1:1000 to the blocking buffer for two days (1ml/slide in a gently agitating sealed humid chamber). Following rinses, secondaries antibodies anti-mouse-alexa-488 (IgG (H+ L) goat, Abcam, ab150113) and anti-rabbit-alexa-594 (IgG (H+L) goat, Invitrogen, A11037) were added at a concentration of 1:1000 to the blocking buffer for 24h (1ml/slide in a gently agitating sealed humid chamber). Slides were washed in TBS and coverslipped with Vectasheild containing DAPI (H-1200, Vector Labs),

sealed with nail polish and imaged. ZEISS Epifluorescence scope was used for image collection with 298 ms exposure among all samples. ImageJ 1.4.3.67 software was used for Aβ plaque and microglia quantification.

## 4.5. STATISTICAL ANALYSIS

All data was collected and analyzed using tools such as Epifluorescence scope, IBM SPSS Statistics 22 , ImageJ, Matlab, and Prism8 for some graph designs. The level of statistical significance was $p < 0.05$ for all tests. The results were presented as the mean ± SEM. Factorial analysis of variance (ANOVA) was used because there were more than two independent variables (sex, metformin and sugar) that could influence dependent variable (performance in behavioural tasks or histology).

### 4.5.1. RESULTS

#### 4.5.1.1.Novel Object Recognition Test

Spatial probability distribution was scored using Matlab heat map measurement (Fig.4), which then was analyzed as probability investigation ratio. The heat map results can be interpreted by the color intensity with dark red indicating that a mouse spent more time in the particular area. Based on the Matlab tracking program, object exploration area was within 5 cm radius from the object where the mouse body centroid was identified. In this measurement, if mice spent equal time investigating the objects, results express it as 1, if mice spent more time investigating the new object, results are greater than 1 and if mice spent more time investigating the old object then results are less than 1. Probability investigation distribution ratio results were (Fig.5): sugar $F(1,22) = .813$, $p \leq .377$; metformin $F(1,22) = .028$, $p \leq .869$; sex $F(1,22) = .048$, $p \leq .829$, between sugar and

metformin $F(1,22) = .271$, $p \leq .608$, metformin and sex $F(1,22) = .024$, $p \leq .877$, sugar and sex $F(1,22) = .012$, $p \leq .914$, and with a significant interaction between sugar, metformin and sex $F(1,22) = 5398$, $p \leq .030$. Results suggest that females who received just metformin explored novel object more than females receiving sugar and metformin. Whereas, males that received metformin and sugar performed similar to their control group, exploring both objects equally.

In the novel object exploration measurement results indicate (Fig.6): sugar $F(1,22) = .159$, $p \leq .693$; metformin $F(1,22) = .548$, $p \leq .467$; sex $F(1,22) = 1.287$, $p \leq .269$, with an interaction between sugar and metformin $F(1,22) = 1.357$, $p \leq .257$, metformin and sex $F(1,22) = .969$, $p \leq .336$, sugar and sex $F(1,22) = .000$, $p \leq .982$, and sugar, metformin and sex $F(1,22) = .295$, $p \leq .592$. The results imply that sugar, metformin and sex did not affect the novel object exploration measure in the AD mouse model of interest.

In the same test, old object exploration time results indicate (Fig.6): sugar $F(1,22) = .733$, $p \leq .401$; metformin $F(1,22) = 3.673$, $p \leq .068$; sex $F(1,22) = .014$, $p \leq .907$, with a significant interaction between sugar and metformin $F(1,22) = 5.870$, $p \leq .024$, metformin and sex $F(1,22) = 1.042$, $p \leq .318$, sugar and sex $F(1,22) = .570$, $p \leq .458$, and sugar, metformin and sex $F(1,22) = .706$, $p \leq .410$. These results suggest that both females and males who received just sugar spent an increased number of times exploring old object, whereas those receiving sugar with metformin spent nearly half of that time.

Discrimination index results were (Fig.6): sugar $F(1,22) = .796$, $p \leq .760$; metformin $F(1,22) = .096$, $p \leq .760$; sex $F(1,22) = .415$, $p \leq .526$, with an interaction between sugar and metformin $F(1,22) = .223$, $p \leq .641$, metformin and sex $F(1,22) =$

.047, $p \leq .830$, sugar and sex $F(1,22) = .147$, $p \leq .705$, and sugar, metformin and sex $F(1,22) = .014$, $p \leq .907$. The results imply that sugar, metformin, and sex do not significantly affect the discrimination index.

In the measurement of distance traveled, results were (Fig.7): sugar $F(1,22) = .016$, $p \leq .900$; metformin $F(1,22) = .204$, $p \leq .656$; sex $F(1,22) = 2.153$, $p \leq .156$, with an interaction between sugar and metformin $F(1,22) = .574$, $p \leq .457$, metformin and sex $F(1,22) = .302$, $p \leq .588$, sugar and sex $F(1,22) = .518$, $p \leq .479$, and sugar, metformin and sex $F(1,22) = .977$, $p \leq .334$. The results imply that sugar, metformin, and sex do not significantly affect the distance traveled.

In stops made results were (Fig.8): sugar $F(1,22) = .619$, $p \leq .440$; metformin $F(1,22) = .424$, $p \leq .522$; sex $F(1,22) = .896$, $p \leq .354$, with an interaction between sugar and metformin $F(1,22) = 2.554$, $p \leq .124$, metformin and sex $F(1,22) = .460$, $p \leq .505$, sugar and sex $F(1,22) = .108$, $p \leq .746$, and sugar, metformin and sex $F(1,22) = .621$, $p \leq .439$. The results imply that sugar, metformin, and sex do not significantly affect the stops.

In this test statistically significant effects were on the probability investigation ratio between sex, sugar and metformin $p \leq .030$, indicating that females who received sugar with metformin treatment spent more time investigating old object compared to males who spent about the same time investigating both objects equally. Another significant effect was on the old object exploration time between sugar and metformin $p \leq .024$. This suggested that mice receiving sugar were impaired on the novel object recognition, whereas metformin improved their exploration time. Although not statistically significant, a notable difference was between mice who received sugar on

distance traveled and stops made - even though mice receiving sugar traveled less in distance, they made more stops on average suggesting that they might have been more anxious.

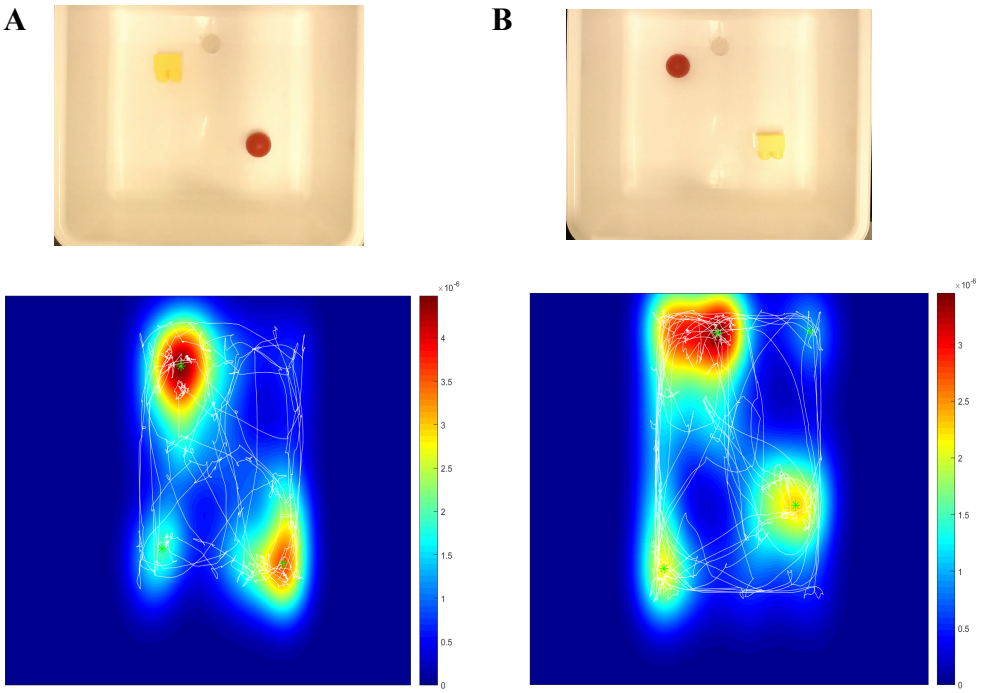



Figure 4. Heat Map of NOR. Location of novel and old objects was counterbalanced to avoid spatial location preference. A) Heat map shows spatial probability of one mouse from sugar group. This mouse spent more time investigating old object. B) Heat map shows spatial probability of investigation by one mouse from sugar with metformin group. This mouse spent more time investigating novel object.

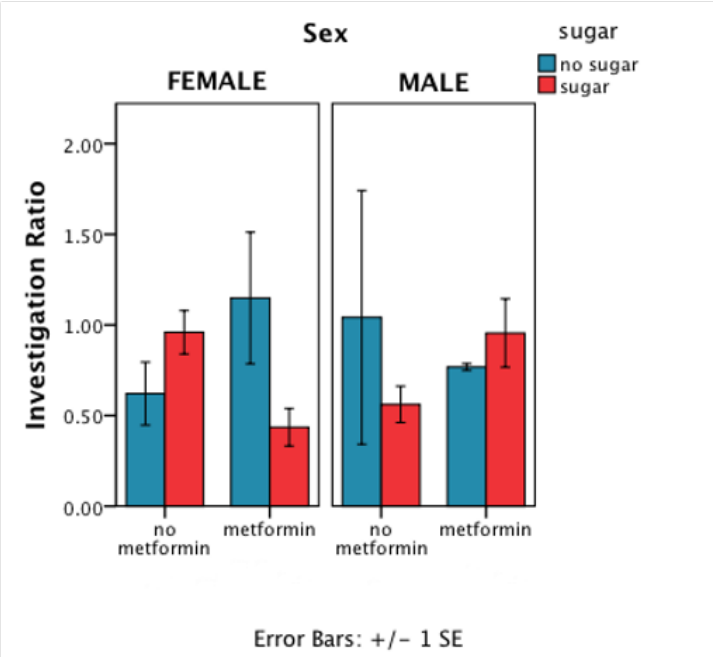
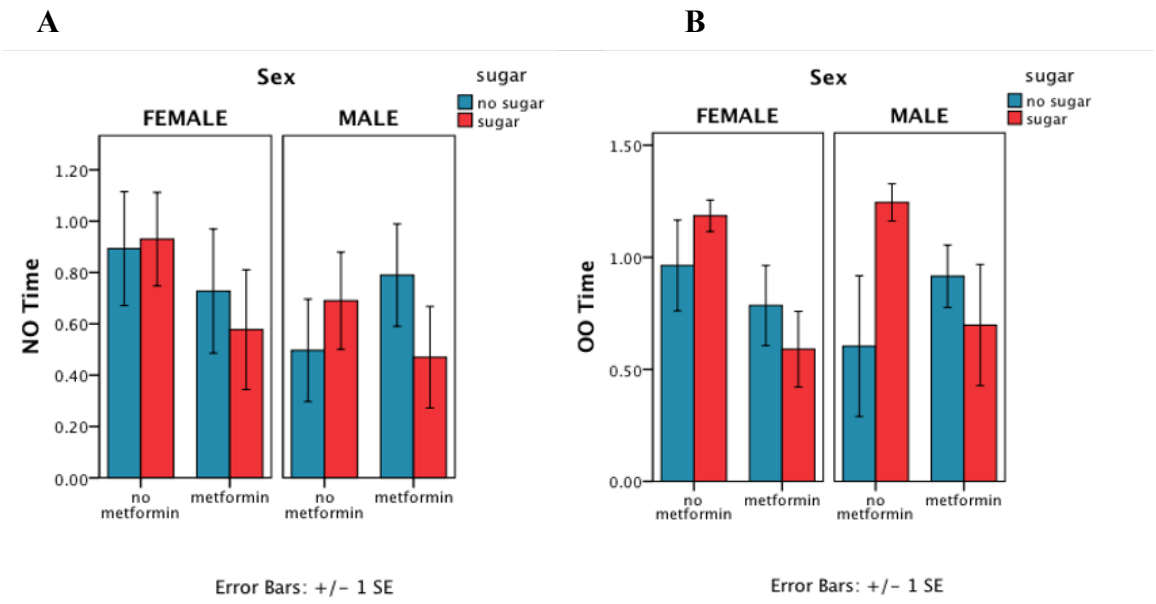



Figure 5. Probability Investigation Ratio. This shows the spatial probability distribution, in other words, probability of mice investigating novel object vs old object. If mice spent equal time investigating the objects, results express it as 1, if mice spent more time investigating the new object, results are greater than 1 and if mice spent more time investigating the old object then results are less than 1. Probability investigation ratio was significant between sugar, metformin and sex ($p \leq 0.030$). It is observed that mice who received sugar spent either equal time exploring both novel and old object or more time exploring old object.



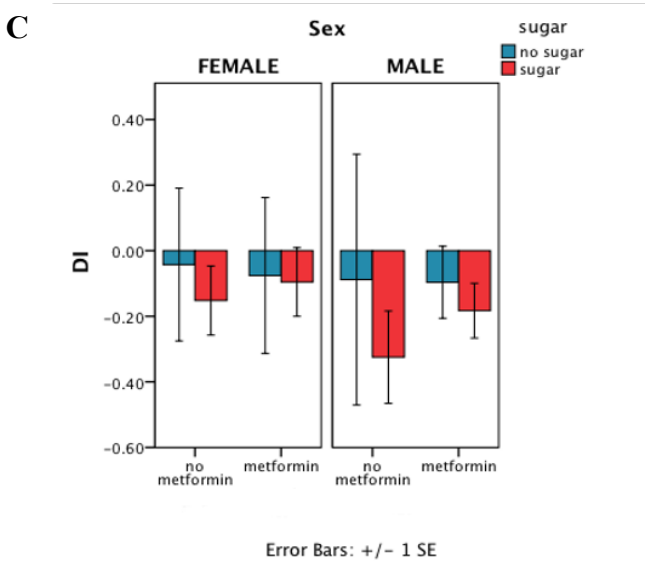



Figure 6. Discrimination Index in NOR. Measurement of increments is in minutes. A) Time spent investigating novel object, in which there was no significant difference between groups. B) Time spent investigating old object, in which there was a significant difference between mice receivingmetformin and mice receiving sugar ($p \leq 0.024$). C) DI shows higher preference for old object, although not statistically significant.

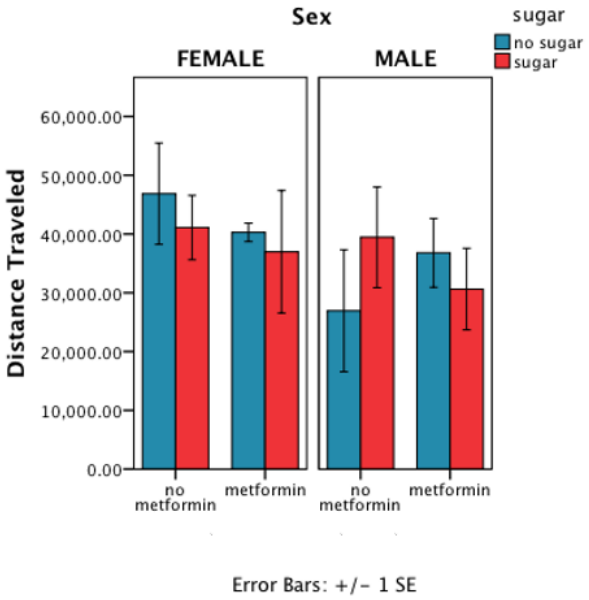


Figure 7. Distance Traveled in NOR. Measurement of increments is in pixels. This measurement was taken of the mean distance traveled between all stops. There was no significant interaction between the groups.

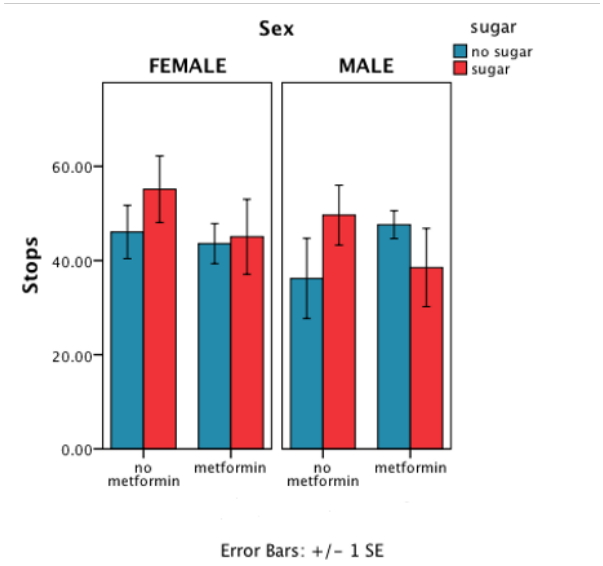



Figure 8. Mean Stops in NOR. This measurement shows the mean of the number of stops among all groups. Statistical analysis shows no significant difference.

#### 4.5.1.2. Balance Beam Test

For this test there were multiple measures completed to score performance of each mice. These measures were walking score, foot slips, and falls. Walking score was measured by observation of hind paw placement on the beam (Fig.9) for nine consecutive steps. In particular, score 0 was given when a mouse placed its hind paw right on top of the beam; score 1 was given if the paw was placed slightly off the beam; 2 was given if the hind paw was placed right on the side of the beam; and score 3 was given if there was a foot slip; while 4 was the lowest score and given if mice slipped with both hind paws. On the walking score measurement results were (Fig. 10): sugar $F(1,22) = .006$, $p \leq .938$; metformin $F(1,22) = 1.976$, $p \leq .174$; sex $F(1,22) = .002$, $p \leq .969$, with an interaction

between sugar and metformin $F(1,22) = .002$, $p \leq .969$, metformin and sex $F(1,22) = .566$, $p \leq .460$, sugar and sex $F(1,22) = 3.104$, $p \leq .092$, and sugar, metformin and sex $F(1,22) = .000$, $p \leq .984$. The results suggest that sugar, metformin and sex do not affect the walking score, nor do these variables interact. On the foot slip measurement results were (Fig. 11): sugar $F(1,22) = 1.072$, $p \leq .312$; metformin $F(1,22) = .596$, $p \leq .448$; sex $F(1,22) = .009$, $p \leq .927$, with an interaction between sugar and metformin $F(1,22) = .000$, $p \leq .988$, metformin and sex $F(1,22) = 1.040$, $p \leq .319$, sugar and sex $F(1,22) = .260$, $p \leq .615$, and significant interaction effect between sugar, metformin and sex $F(1,22) = .4.349$, $p \leq .049$. The results suggest that sugar, metformin and sex affect the foot slip score with an interaction of $p \leq .049$ between these variables.

On the average falls measurement (Fig. 12), results indicate: sugar $F(1,22) = .403$, $p \leq .532$; metformin $F(1,22) = 1.333$, $p \leq .261$; sex $F(1,22) = 1.333$, $p \leq .261$, with an interaction between sugar and metformin $F(1,22) = .333$, $p \leq .570$, metformin and sex $F(1,22) = .563$, $p \leq .461$, sugar and sex $F(1,22) = .213$, $p \leq .649$, and sugar, metformin and sex $F(1,22) = .963$, $p \leq .337$. The results suggest that sugar, metformin and sex do not affect the falls, nor do these variables interact.

In this test, sugar, metformin and sex interaction affected only foot slip measurement and did not affect any other aspects of performance that were measured. If these measurements were significant, it would suggest that sugar impaired motor functions and balance while metformin improved it, however, this was not the case. Although, the foot slip measurement was with statistically significant interaction effect, it was contrary to our hypothesis.

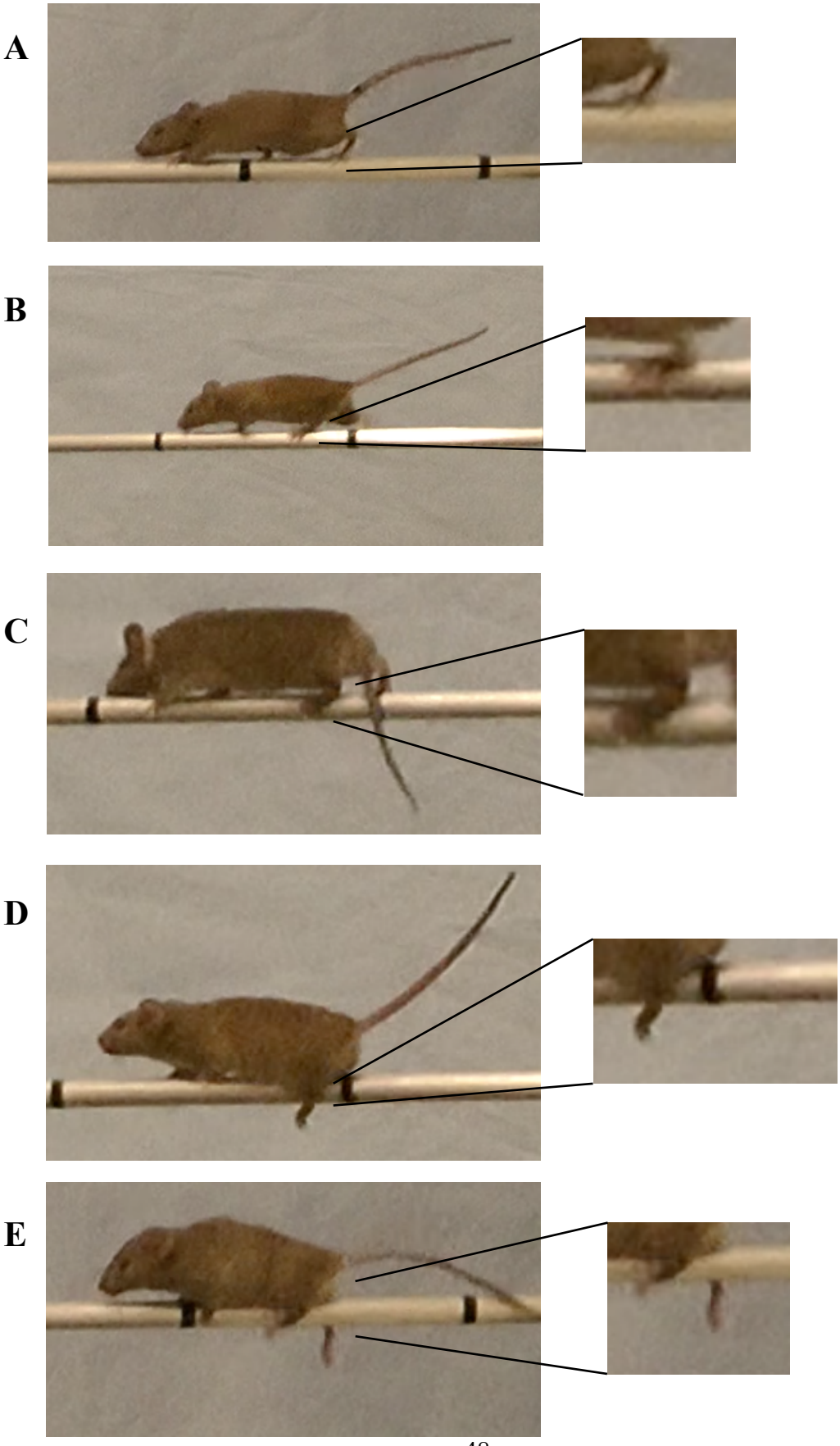

A
B
C
D
E

Figure 9. Balance-Beam Test walking score. (A) Score 0 was given when a mouse placed its hind paw right on top of the beam; (B) score 1 was given if the paw was placed slightly off the beam; (C) score 2 was given if the hind paw was placed right on the side of the beam; (D) score 3 was given if there was a foot slip; (E) score 4 was given if mice slipped with both hind paws.

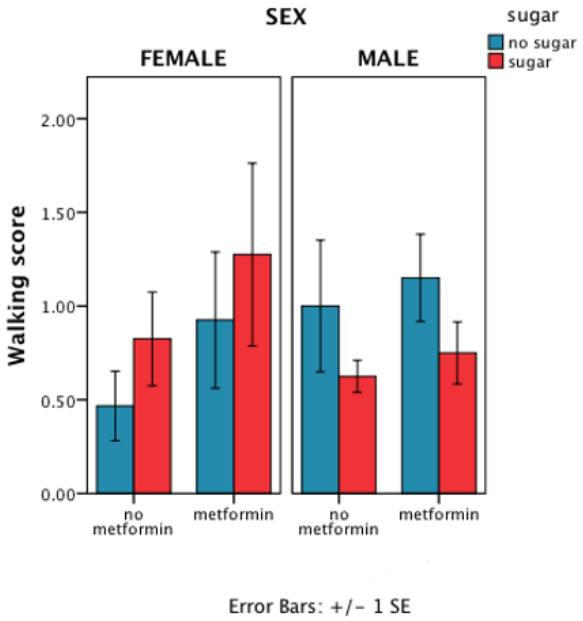



Figure 10. Mean Walking Score on BBT. This measurement shows the average score on walking across the beam on trial 3. The lower the score, the better performance. There was no statistically significant difference between the groups.

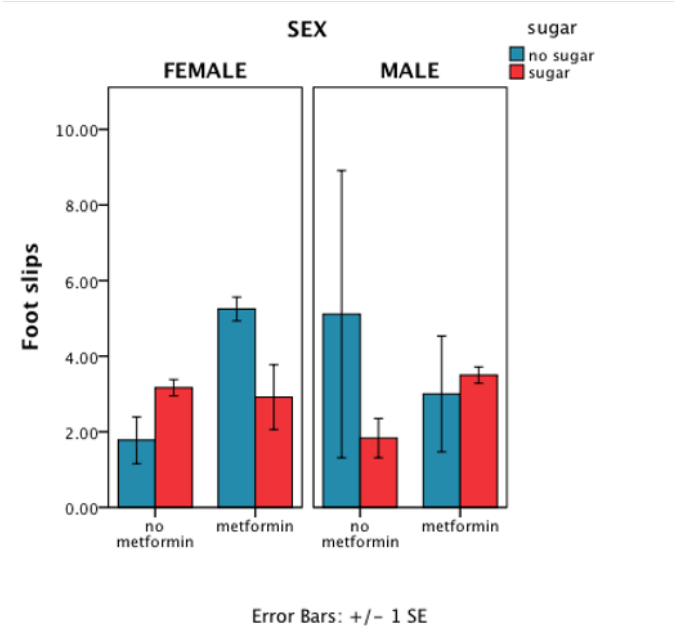


Figure 11. Mean Number of Foot Slips on BBT. This score was measured among all mice across three successful trials. Statistically significant difference was observed between sex, sugar and metformin interaction with ($p \leq 0.049$). Results showed better performance among those mice who did not receive sugar.

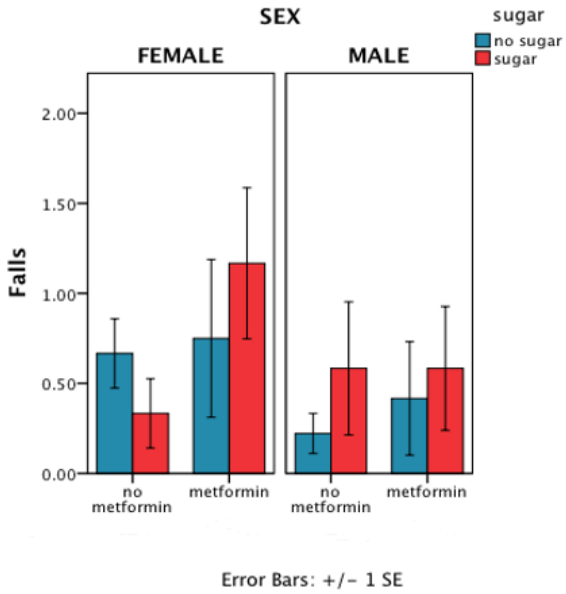



Figure 12. Mean Falls on BBT. Number of falls measured among four groups. There was no significant interaction effect, however, there is a trend for mice receiving sugar to fall more.

#### 4.5.1.3.Context Fear Conditioning Test

The fear conditioning procedure involved two environments. One was context A (where mice received a mild foot shock) and the other was novel context B, never associated with foot shock, which was a different shape box in a novel room.

In context A, the stops results were (Fig.13): sugar $F(1,22) = .539$, $p \leq .470$; metformin $F(1,22) = 1.347$, $p \leq .258$; sex $F(1,22) = .189$, $p \leq .668$, with an interaction between sugar and metformin $F(1,22) = .028$, $p \leq .868$, metformin and sex $F(1,22) = .040$, $p \leq .843$, sugar and sex $F(1,22) = 1.176$, $p \leq .290$, and sugar, metformin and sex

$F(1,22) = .224$, $p \leq .641$. The results imply that sugar, metformin, and sex do not significantly affect the number of stops.

In distance traveled results were (Fig.13): sugar $F(1,22) = .020$, $p \leq .888$; metformin $F(1,22) = 2.788$, $p \leq .109$; sex $F(1,22) = .003$, $p \leq .956$, with an interaction between sugar and metformin $F(1,22) = .034$, $p \leq .855$, metformin and sex $F(1,22) = .037$, $p \leq .849$, sugar and sex $F(1,22) = 1.539$, $p \leq .228$, and sugar, metformin and sex $F(1,22) = .037$, $p \leq .849$. The results imply that sugar, metformin, and sex do not significantly affect the distance traveled.

In the freezing measurement results were (Fig.14): sugar $F(1,22) = .139$, $p \leq .713$; metformin $F(1,22) = 2.457$, $p \leq .131$; sex $F(1,22) = .005$, $p \leq .945$, with an interaction between sugar and metformin $F(1,22) = .024$, $p \leq .879$, metformin and sex $F(1,22) = .097$, $p \leq .759$, sugar and sex $F(1,22) = 1.397$, $p \leq .250$, and sugar, metformin and sex $F(1,22) = .009$, $p \leq .927$. The results imply that sugar, metformin, and sex do not significantly affect the freezing measure.

In the speed traveled results were (Fig.15): sugar $F(1,22) = .081$, $p \leq .778$; metformin $F(1,22) = 2.313$, $p \leq .143$; sex $F(1,22) = .000$, $p \leq .991$, with an interaction between sugar and metformin $F(1,22) = .001$, $p \leq .974$, metformin and sex $F(1,22) = .044$, $p \leq .836$, sugar and sex $F(1,22) = 1.552$, $p \leq .226$, and sugar, metformin and sex $F(1,22) = .000$, $p \leq .985$. The results imply that sugar, metformin, and sex do not significantly affect the speed measure.

In context B (novel context) on measurement stops made results were (Fig.16): sugar $F(1,22) = 2.530$, $p \leq .126$; metformin $F(1,22) = .646$, $p \leq .430$; sex $F(1,22) = .019$, $p \leq .893$, with an interaction between sugar and metformin $F(1,22) = .457$, $p \leq .506$, and

significant interaction between metformin and sex $F(1,22) = 13.545$, $p \leq .001$, sugar and sex $F(1,22) = 7.651$, $p \leq .011$, and sugar, metformin and sex $F(1,22) = 1.530$, $p \leq .126$. The results imply that interaction between metformin and sex, as well as sugar and sex significantly affected the number of stops.

In distance traveled measurement results were (Fig.16): sugar $F(1,22) = 1.269$, $p \leq .272$; metformin $F(1,22) = .258$, $p \leq .617$; sex $F(1,22) = .003$, $p \leq .958$, with an interaction between sugar and metformin $F(1,22) = .228$, $p \leq .638$, and significant interaction between metformin and sex $F(1,22) = 8.683$, $p \leq .007$, sugar and sex $F(1,22) = 8.513$, $p \leq .008$, and sugar, metformin and sex $F(1,22) = 2.811$, $p \leq .108$. The results imply that interaction between metformin and sex $p \leq .007$, as well as sugar and sex $p \leq .008$ significantly affected distance traveled in novel context.

Freezing measurement in novel context results were (Fig.17): sugar $F(1,22) = 2.389$, $p \leq .136$; metformin $F(1,22) = .285$, $p \leq .599$; sex $F(1,22) = .025$, $p \leq .877$, with an interaction between sugar and metformin $F(1,22) = .360$, $p \leq .555$, and significant interaction between metformin and sex $F(1,22) = 12.989$, $p \leq .002$, sugar and sex $F(1,22) = 8.757$, $p \leq .007$, and sugar, metformin and sex $F(1,22) = 3.400$, $p \leq .079$. The results imply that interaction between metformin and sex $p \leq .002$, as well as sugar and sex $p \leq .007$ significantly affected freezing time.

In speed measurement results were (Fig.18): sugar $F(1,22) = 1.356$, $p \leq .257$; metformin $F(1,22) = .268$, $p \leq .610$; sex $F(1,22) = .004$, $p \leq .948$, with an interaction between sugar and metformin $F(1,22) = .188$, $p \leq .669$, and significant interaction between metformin and sex $F(1,22) = 9.284$, $p \leq .006$, sugar and sex $F(1,22) = 8.337$, $p \leq .009$, and sugar, metformin and sex $F(1,22) = 2.652$, $p \leq .118$. The results imply that

interaction between metformin and sex $p \leq .006$, as well as sugar and sex $p \leq .009$ significantly affected the average speed mice traveled.

Minute by minute stop measurement in both environments was displayed (Fig.19), which shows mice response on stopping across a five-minute interval. It is observed that groups that received sugar or sugar with metformin had fewer stops compared to other groups. However, after a third minute there was an increase in stops made, which makes us to wonder if mice displayed increased anxiety towards the end, while other groups became more comfortable to the environment.

In this test statistically significant effects were in stops made in the novel context between metformin and sex, $p \leq .001$, and sugar and sex, $p \leq .011$, suggesting that sugar increased the number of stops made only in males. Also, males who received metformin traveled more in distance and had more stops made than males who did not receive metformin. Whereas, females who received sugar or metformin traveled less in distance and had less stops made in the novel context. In addition, females who received sugar with metformin traveled about the same distance in both context A and context B, showing that sugar impaired their ability to differentiate both contexts. Other statistically significant results were in freezing in the novel context between metformin and sex, $p \leq .002$, sugar and sex, $p \leq .007$. Sugar increased the freezing time for females, and metformin decreased the freezing time for males. Finally, significantly statistical effect was on the speed measurement between metformin and sex, $p \leq .006$, sugar and sex, $p \leq .009$. Results suggest that males who received sugar traveled with higher speed compared to females. As well as, males who received metformin had higher speed compared to the control.

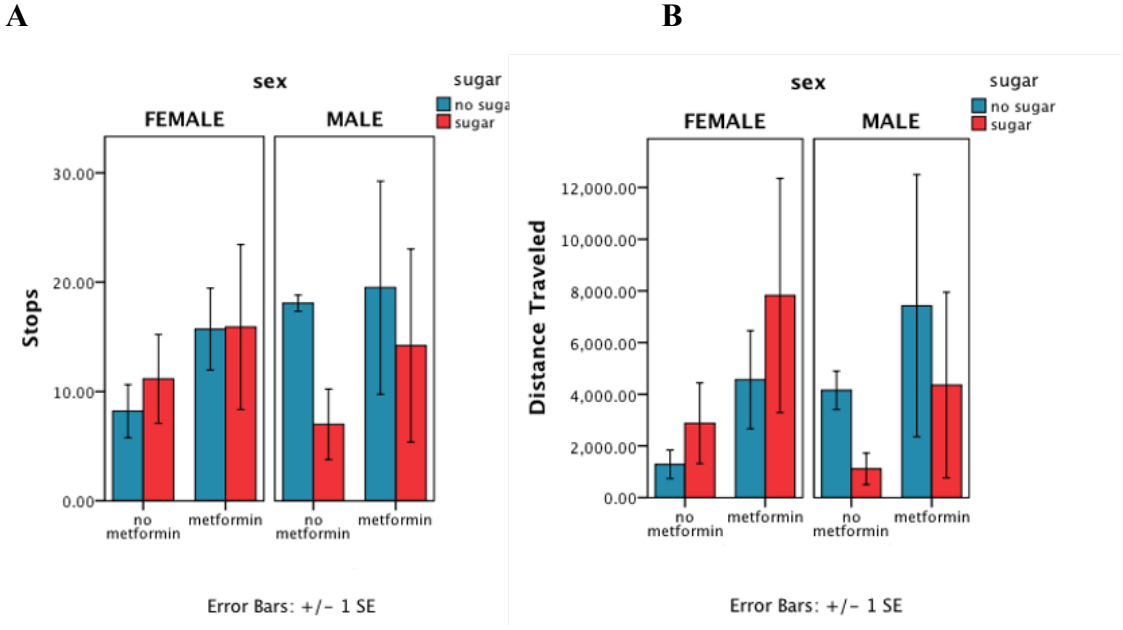



Figure 13. FC Context Stops and Distance Traveled.
A) Number of stops made in context A among all mice.
B) Distance traveled in increments of pixels in context
A among all mice. In both of these measurements there was
no significant interaction effect between sugar, metformin or sex.

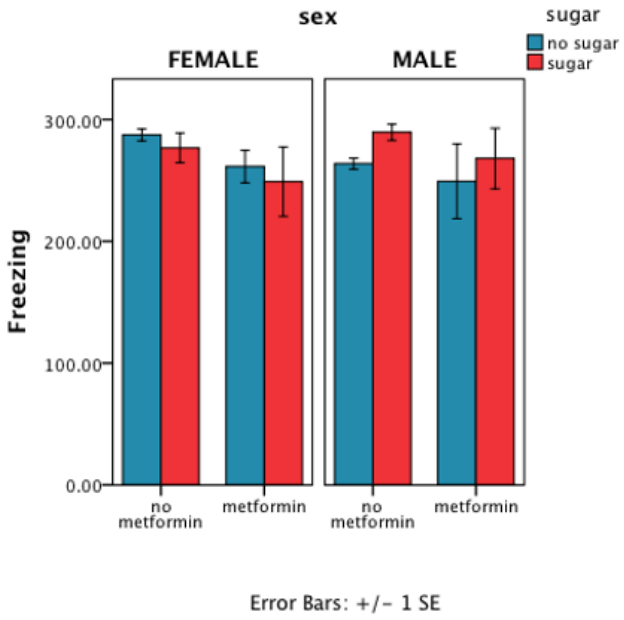



Figure 14. Freezing in Context A. This measurement shows
immobility in increments of seconds, also called the freezing
time among all groups. There was no significant
interaction effect in this measurement in context A.

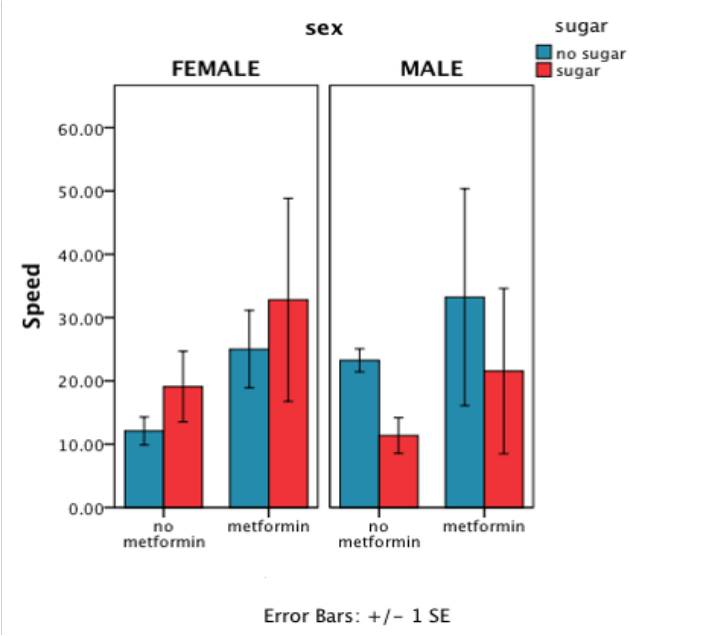
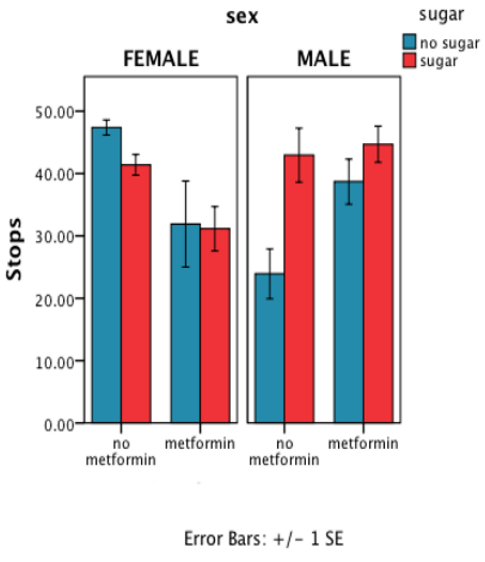



Figure 15. Mean Speed in Context A. This measurement calculates average speed with which mice were traveling in increments of seconds per pixel in context A. There was no statistical interaction effect between sugar, metformin or sex.

**A**




**B**

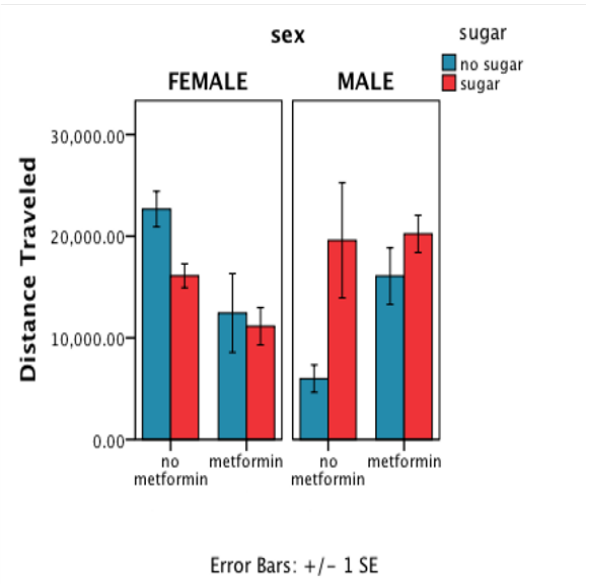


Figure 16. FC Novel Context Stops and Distance Traveled. A) Number of stops made in novel context with significant difference between sex and sugar ($p \leq 0.011$), and sex and metformin ($p \leq 0.001$). B) Distance traveled in increments of pixels in novel context with significant difference between sex and metformin ($p \leq 0.007$), as well as sex and sugar ($p \leq 0.008$)

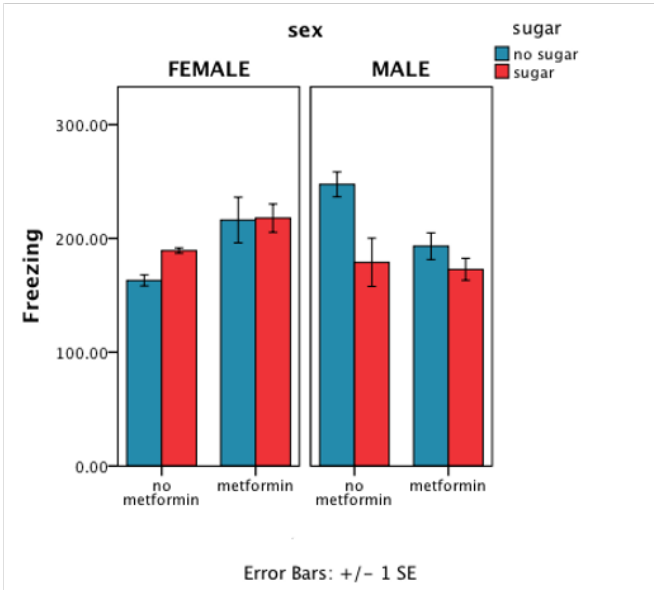



Figure 17. Freezing Time in Novel Context. This measurement was scored by time spent (seconds) in immobility during a five-minute period. There was a significant interaction between sex and metformin ($p \leq 0.002$), and sex and sugar ($p \leq 0.007$)

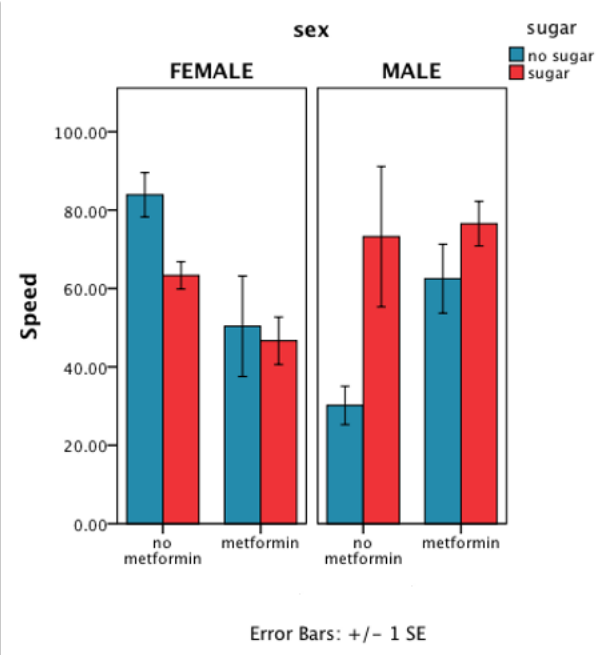


Figure 18. Mean Speed in Novel Context. This measurement shows speed in increments of seconds per pixel with which mice traveled in context B. There is a significant interaction between sex and metformin ($p \leq 0.006$), and sex and sugar ($p \leq 0.009$).

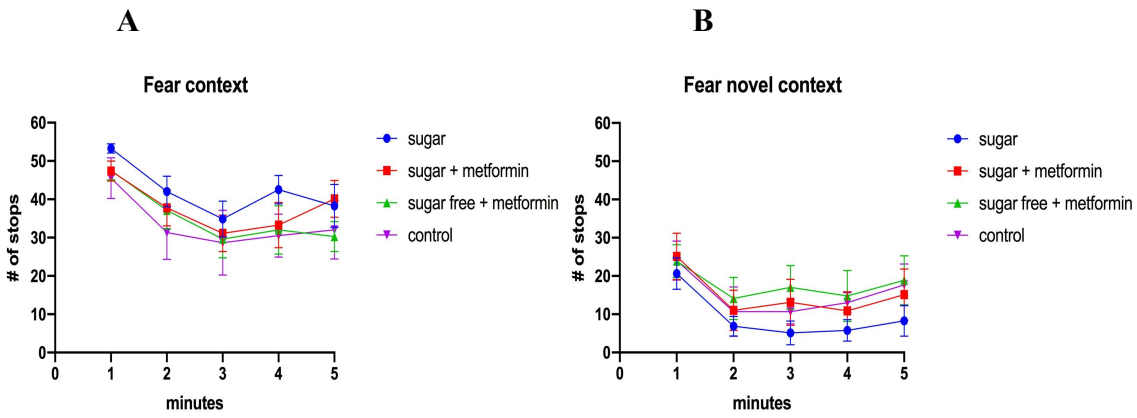



Figure 19. Minute by Minute Stops. This measurement shows the number of stops in FC task. A) Number of stops in context A. B) Number of stops in context B (novel context).

#### 4.5.1.4. Histology Results

Deposition of Aβ plaques were measured in one coronal brain section per mouse. Regions of interest included the left and right neocortex with retrosplenial area, posterior parietal association areas, primary somatosensory area and left/right hippocampus at 4 months and 13 days mice were perfused and their brains used for histology.

In the number of plaques measurement results were (Fig.20): sugar $F(1,22) = 4.628$, $p \leq .007$; metformin $F(1,22) = 2.513$, $p \leq .004$; sex $F(1,22) = 5.395$, $p \leq .030$, with an interaction between sugar and metformin $F(1,22) = 4.703$, $p \leq .041$, metformin and sex $F(1,22) = 1.988$, $p \leq .173$, sugar and sex $F(1,22) = .142$, $p \leq .710$, and sugar, metformin and sex $F(1,22) = .078$, $p \leq .783$). In terms of inflammation observed in the brain, intensity of microglia activity was calculated using ImageJ. Inflammation intensity

measurement results were (Fig.21): sugar $F(1,22) = .995$, $p \leq .329$; metformin $F(1,22) = .014$, $p \leq .907$; sex $F(1,22) = .054$, $p \leq .819$, with an interaction between sugar and metformin $F(1,22) = 7.870$, $p \leq .010$, metformin and sex $F(1,22) = .263$, $p \leq .613$, sugar and sex $F(1,22) = 1.416$, $p \leq .247$, and sugar, metformin and sex $F(1,22) = .054$, $p \leq .818$.

Results suggest that females had more plaques than males, plaque deposition was increased with sugar and decreased with sugar and metformin treatment. In terms of inflammation in the brain, there were no sex differences, but sugar increased inflammation and sugar with metformin decreased it.

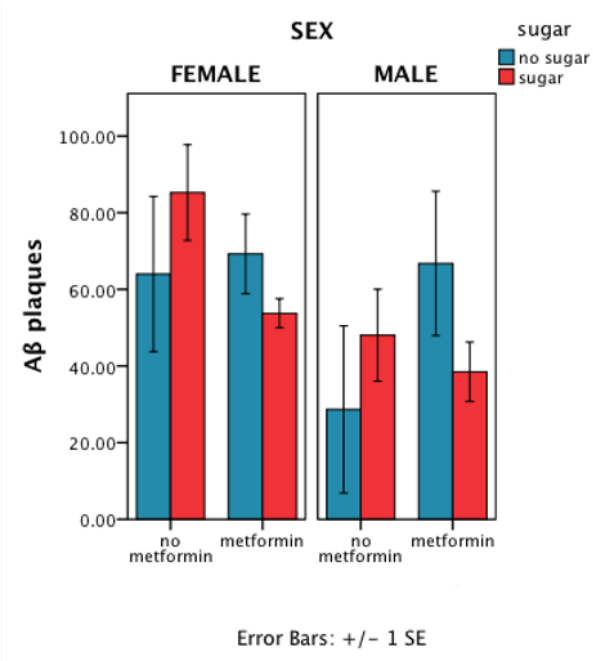



Figure 20. Mean Distribution of Number of Aβ plaques. There was a significant difference between sexes ($p \leq 0.030$) and metformin and sugar interaction ($p \leq 0.041$). Higher deposition of plaques was among females and mice that were given sugar with metformin treatment had decreased number of plaques.

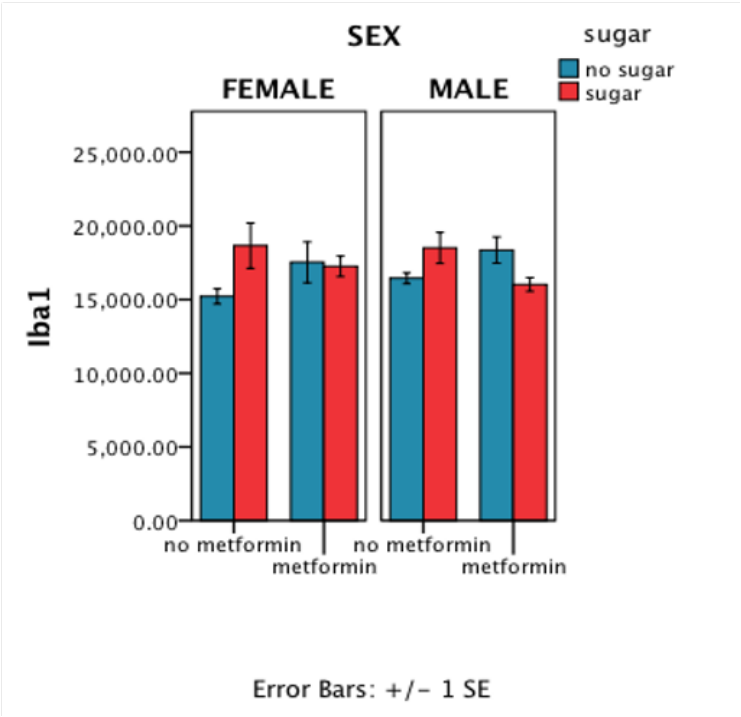
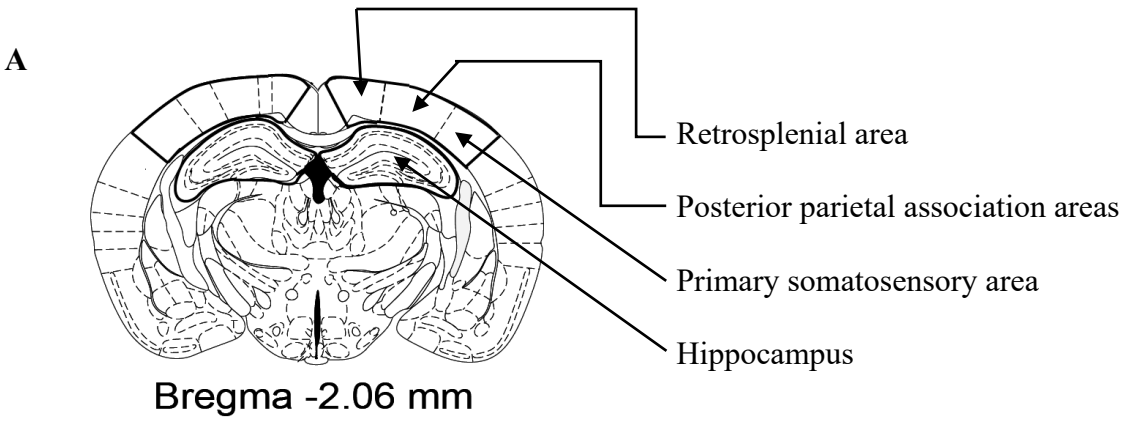



Figure 21. Inflammation in the Brain. There was a significant difference between sugar and metformin interaction ($p \leq 0.010$). Sugar increased inflammation in the brain and sugar with metformin decreased inflammation



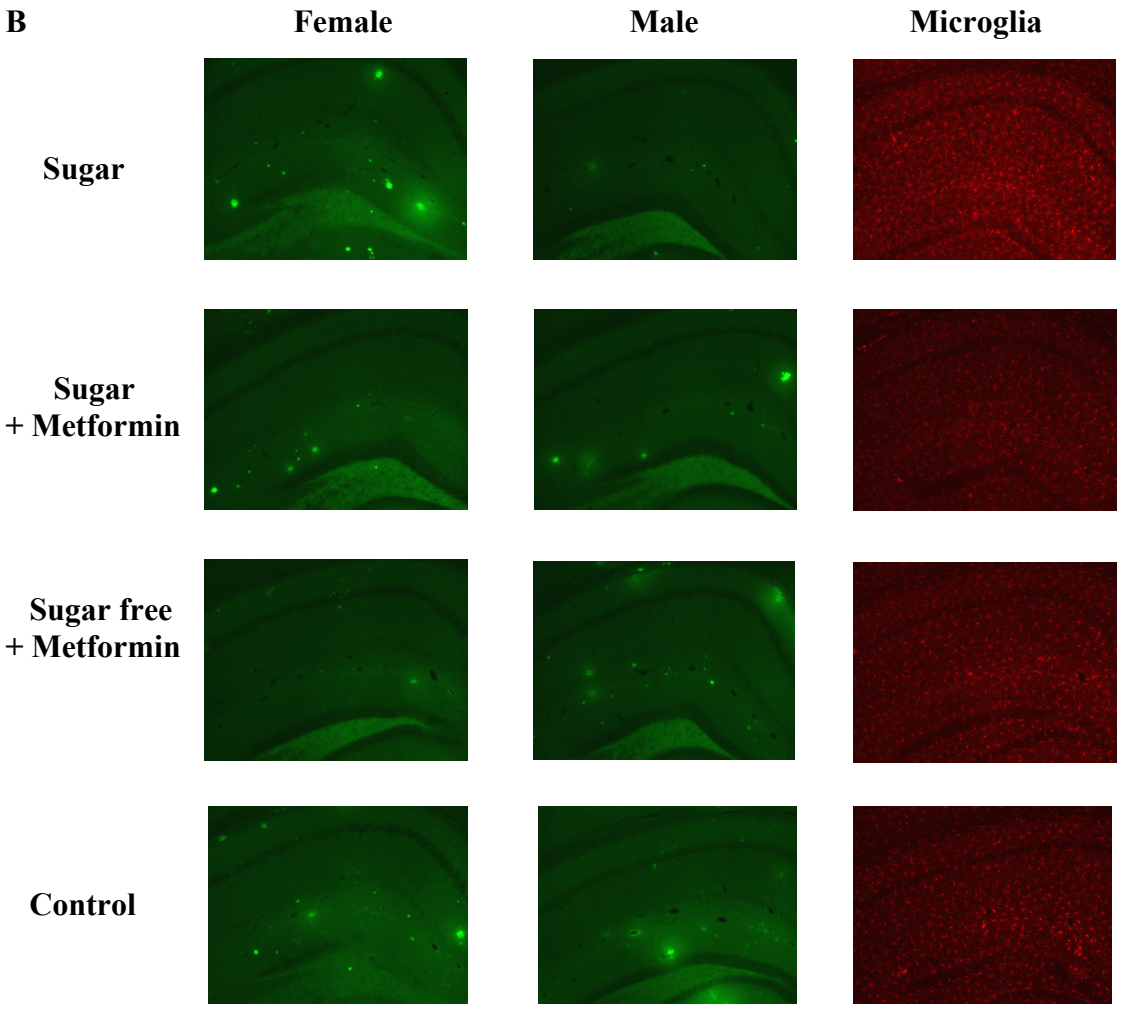



Figure 22. Region of Interest for Histology.
A) Coronal mice brain sections, corresponding with bregma ~2.06 mm, were chosen to quantify Aβ plaques. Region of interest was left and right cerebral Isocortex with retrosplenial area, posterior parietal association areas, primary somatosensory area and left/right hippocampus. B) Left hippocampus area among males and females in each group. It can be noticed that females show more plaques (in green), especially in sugar groups. In red is shown the microglia intensity, which signifies inflammation of the left hippocampus.

## CHAPTER 5: GENERAL DISCUSSION

The main hypothesis of this study was that AD pathology is importantly regulated by sugar metabolism in the brain. Therefore, our major predictions were that high dietary

sugar would aggravate AD pathology, while metformin could ameliorate AD pathology. The results from histology confirmed the hypotheses, suggesting statistically significant effect on Aβ plaque deposition. Sugar increased the quantity of Aβ plaques, while metformin with sugar normalized Aβ plaques. In fact, females had more plaques in the brain than males. Also, histology on inflammation revealed statistically significant effects with no sex differences observed, showing that sugar increased inflammation in the brain, while metformin with sugar treatment decreased it.

The behavioural test results showed no consistent effects of sugar or metformin among all measurements. These results disconfirm the hypothesis. There could have been other factors that impacted behavioural test results such as choice of experimental design for behavioural tests or mouse strain characteristics to not develop behavioural impairments as early as four months of age. Although the histology results are clear and confirm that sugar made AD pathophysiology worse (and metformin reversed these effects), the changes in plaque and inflammation did not translate into behavioural impairment. Behavioural changes in AD may relate to some other changes in the brain. The dissociation between Aβ and inflammation effects and behavioural results can be due to at least two possibilities. First, Aβ and inflammation may not directly affect behaviour at four and a half months old mice in this particular AD model or, second, our behavioural measures may not have been as sensitive to sugar and metformin as our brain measures.

In the present study, APP/PS1 mouse models of Alzheimer's disease were used to examine effects of sugar on severity of AD pathology, while investigating if metformin can delay or reverse AD pathology. The APP/PS1 mouse model is based on the familial

type of AD and expresses EOD. It is important to recognize that our results might be limited to just this form of AD pathology, strongly driven by EOD genes. If we had employed a model more similar to later onset, sporadic AD, the most common form in humans, we may have observed a different pattern of results.

Our most important statistically significant results were found in histology, which showed that females had more AB plaques. Equally important, there was a significant interaction between sugar and metformin. In particular, mice who received sugar had increased plaque deposition and mice who received sugar with metformin had decreased plaque deposition. This means that metformin may have ameliorated plaque development. Similarly, increased microglia intensity was observed in mice that received sugar, which signifies inflammation in the brain. Meanwhile, mice receiving sugar with metformin had decreased inflammation with reduced levels of microglia intensity.

The present study confirms that females show increased plaque deposition in the brain compared to males, which was suggested by other studies as well (Wang et al., 2003).

Sex differences in behavioural tasks may be due to hormonal differences. Researchers Abbott, Morris, Westbrook and Reichelt (2016) studied sex-specific effects of high sugar intake on spatial memory performance in rats. They gave rats two-hour daily exposure to sucrose for 14 days before testing on place and object-in-place recognition tasks. Their findings indicated that female rats exposed to sugar performed better on the object-in-place task when their estrogen levels were high, suggesting that while hippocampus may be impaired due to the high sugar exposure, increased estrogen may have boosted neuroplasticity thus enabling spatial memory encoding. Their study also found that both

male and female rats exposed to sucrose were impaired in the place recognition task. In conclusion, Abbott et al. (2016) states that high sugar diets may lead to worse cognitive deficits in males and females, after menopause.

These test results and histology lead to the conclusion that metformin is effective if interacting with sugar and possibly it can control glucose disposition in the brain. In contrast, the behavioural test results do not show the same positive interaction when metformin is consumed with sugar free pudding. It might be that groups which received sugar chocolate puddings had developed insulin resistance in the brain and metformin ameliorated it, as suggested by other studies, such as Cao et al. (2007) and de la Monte, (2014). Conversely, sugar free chocolate pudding could potentially cause different toxic changes that metformin could not stop. It has been suggested by other studies that artificial sweeteners could lead to metabolic changes and increase risk for negative health outcomes (Swithers, 2016).

There were some limitations to the study. First, it would be better for statistical analysis and general conclusions to have a larger sample size and group size. Possibly, by adding two more groups of the same APP/PS1 mouse model we could identify if sugar free chocolate pudding treatment has significant difference between the group that does not receive any treatment but still holds the AD mutation genes. From two additional groups, one would receive just metformin that would not be mixed with any additional diets and the other would be control in which mice would not receive any type of treatment. It would be meaningful to add wild-type animal group with no genome mutations. Another limitation is the timeline of experimental design. If treatment could have been extended and perfusions done later than four-and-a-half months we might

observe different results. According to the Jackson laboratory genetic data, these mice develop amyloid beta deposits in the brain by six to seven months of age. Other studies show plaque deposition in this particular mouse model at nine months of age (Wang et al., 2003), while Alzforum website reports this mice model showing plaque deposition at three months of age. However, this study showed that there were significant plaques in the brains at four-and-a-half  months of age. This suggests that high sugar diet and artificial sweeteners could have increased the progression of AD at an earlier stage. Choosing additional behavioural tests and assessing them later than what was initially done, could potentially lead to different test results.

Even though there are some limitations, we can still consider the effect of diet on AD as an important factor. Although sugar increased plaque deposition and inflammation in the brain, the sugar free diet still showed meaningful results that can suggest impairment due to the diet. Therefore, sugar free, which is artificially sweetened, does not necessarily mean better or healthier because there may be many additives that are toxic and unhealthy to the body and brain.

For people at increased risk for AD or those who want to possibly prevent it, they should decrease intake of foods that disrupt insulin and blood sugar balance. That means avoiding processed foods and beverages high with added sugars, and eating whole, unrefined foods with natural fats, such as Omega 3, which is important for brain health, especially fish, nuts and seeds, olives and olive oil (Pasinetti & Eberstein, 2008). As Pasinetti & Eberstein (2008) said, if lifestyle changes, including diet, can be a tool for treatment or prevention purposes, then these changes have to be permanent, otherwise temporary changes are not for a long-term benefit.

## 5.1. CONCLUSION AND FUTURE DIRECTIONS

In conclusion the findings of this study imply that dietary sugar aggravated Aβ plaques in the brain, with higher plaque deposition among females, meanwhile, metformin ameliorated AD pathophysiology. Sugar also increased inflammation in the brain among both sexes, while metformin decreased it. Based on the results, metformin was the most effective if interacted with sugar, possibly due to the glucose mechanism in the brain. The behavioural test results showed no consistent effects of sugar or metformin among all measurements. In the present study there was an inconsistent performance among the rodents with some outliers present in the data (Fig. 23). Therefore, it might be meaningful to look at individual subjects and their results in both behavioural and histology assessments. It would be recommended to compare and study dietary sugar to sugar in its original dietary context, which is available in fruits and vegetables. This theory can also translate into human studies by comparing AD patients and their diets and follow their progression of the disease.

When metformin is prescribed to Type 2 diabetes patients, usually it comes with other health related recommendations, such as physical activity and healthy eating. While AD is considered as Type 3 diabetes due to the overlapping symptoms, it still does not answer the question of what causes it and how to treat it. Researchers have just found what are the possible risk factors and what we can do to increase our chances of healthy aging, but we need to find the answers, so we can stop this devastating disease. Further studies are needed with both animal and human subjects, with increased sample size and possible interactions between sugar and metformin. It would be recommended to look at other mouse models of AD that express LOD and would better simulate human disease.

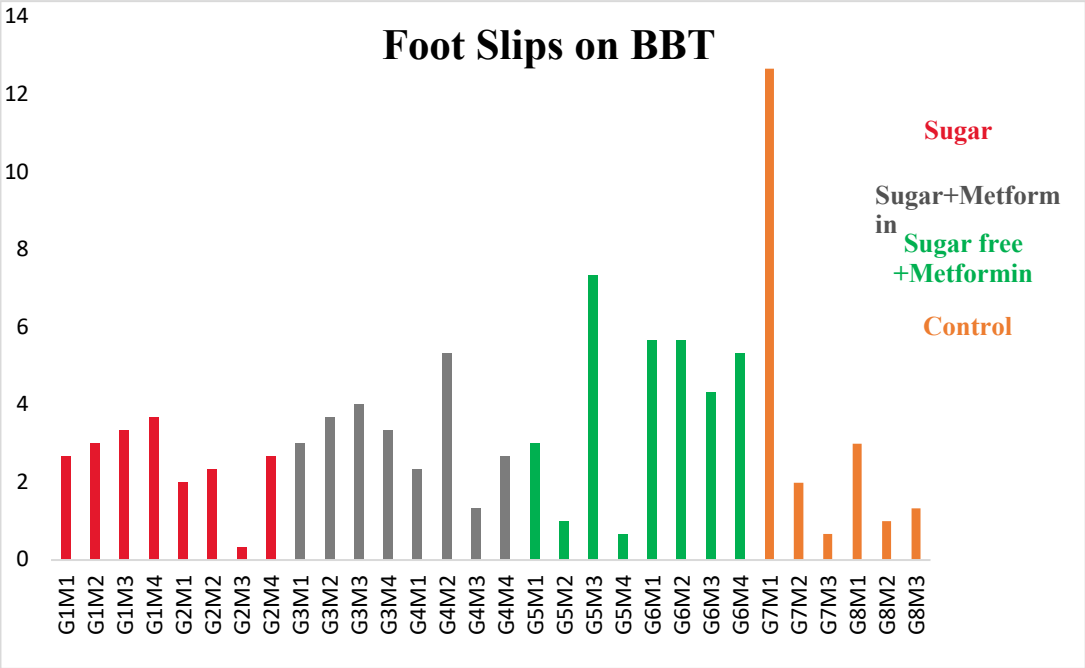



Figure 23. Individual Results on Foot Slips. In this graph we can notice individual differences among all subjects on BBT, foot slip measurement. There is an outlier in control group, which was recognized in statistical data with more than 2 SD from the mean, however, it was not removed due to the consistent data report throughout the analysis. This outlier (averaging with 12-foot slips) was a single housed male and overweight.

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