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The Contribution of a High-fat Diet to
Neurodegeneration and Oxidative Stress in
a Drosophila melanogaster Model of
Alzheimer's Disease

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Abstract:

Alzheimer's disease (AD) poses a growing global health challenge due to its increasing prevalence. AD is a complex neurodegenerative disease characterised by abnormal accumulation of extracellular amyloid beta (A β) plaques and intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated Tau, leading to brain changes and cognitive decline. Predisposing genetic mutations, such as those affecting A β accumulation and aggregation or apolipoprotein signalling, enhance the risk of developing AD which may be further accelerated by environmental factors such as diet.

To investigate the effect of a high-fat (HF) diet on AD pathology a *Drosophila melanogaster* model was used, including flies overexpressing A β arc mutations, resembling familial AD, and hApoE4 expressing flies modelling sporadic AD. The flies were divided into normal food (NF) and high-fat (HF) groups with further grouping by age (10 and 45 days old) and sex. Multiple assays were conducted, characterising oxidative stress markers, cleaved Caspase-3 expression, lifespan and climbing ability.

The findings suggest a HF diet, age, and genotype are all factors that can influence oxidative stress, the response to oxidative stress, and AD pathogenesis. Notably, the use of coconut oil in the HF diet hinted at potential neuroprotective effects, challenging expectations that this diet would exacerbate AD pathogenesis.

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Abbreviations:

AD – Alzheimer's disease

A β - amyloid beta

NFT – neurofibrillary tangle

APP – amyloid precursor protein

AICD – APP intracellular domain

CTF β – C-terminal β fragment

NF – normal food

A β arc – UAS-A β arc

hApoE4 – UAS-hApoE4

ApoE ϵ 4 – Apolipoprotein ϵ 4

LOAD – late onset Alzheimer's disease

EOAD – early onset Alzheimer's disease

A β ₄₂ – A β ₁₋₄₂

SAD – sporadic AD

FAD – familial AD

RS – redox stress

ROS – reactive oxygen species

OS – oxidative stress

mtDNA – mitochondrial DNA

LP – lipid peroxidation

SOD – superoxide dismutase

GM – gut microbiome

GI – gastrointestinal

MDA - malondialdehyde

CSF – cerebrospinal fluid

MCI – mild cognitive impairment

PET – positron emission tomography

CTF α – C-terminal fragment α

SAPP β – soluble β -APP

HF – high-fat

W₁₁₁₈ - wildtype

CNS – central nervous system

GBA – gut-brain axis

LPS – lipopolysaccharides

BBB – blood-brain barrier

BDNF – brain-derived neurotrophic factor

PAMP – pathogen-associate molecular pattern

ER – endoplasmic reticulum

BDSC – Bloomington drosophila stock centre

XO – xanthine oxidase

DI – deionised

TBA – thiobarbituric acid

PFA – paraformaldehyde

PBS – phosphate buffered saline

HL3 – haemolymph-like solution 3

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1 Introduction:

1.1 Alzheimer's Disease

Dementia is an all-encompassing term for a group of symptoms which indicate difficulty in cognitive functioning. Dementia currently affects around 46 million people globally, a figure which is expected to double within the next 20 years due to increased life expectancy (Feringa and van der Kant, 2021). It is estimated that 5-8 % of individuals over age 65, 15-20 % over age 75, and 20-25 % over age 85 are affected by dementia (Cheignon et al., 2018).

AD is the most common form of dementia. The total number of people with AD is expected to reach 74.7 million in 2030 and reach 131.5 million in 2050, classifying this disease as a major global health challenge (Kesika et al., 2021; Feringa and van der Kant, 2021; Wu et al., 2017; Hampel et al., 2021). Due to the increase in cases, and the associated high morbidity, disability, and fatality rates, AD is responsible for significantly increasing the global burden of public health and presents a rising challenge in its treatment and management (Guo et al., 2021).

AD is a biologically complex neurodegenerative disease that has no known single cause, however there are several factors that have been shown to affect its onset. Growing evidence has associated certain risk factors with AD onset and pathology such as hypertension, diabetes, high cholesterol, and oxidative stress and mitochondrial dysfunction (Doifode et al., 2021).

1.1.1 Stages and Symptoms of Alzheimer's Disease

AD is clinically characterised by negative changes in memory, cognitive, and motor functions, which lead to a decline in mental, behavioural, and functional activities (Kesika et al., 2021). Individuals might display symptoms of memory loss, intellectual disabilities, language problems and personality and behavioural changes which occur because of damaged or destroyed neuronal cells in the brain (Buccellato et al., 2021).

Symptoms of AD and other dementias differ from ordinary age-related changes, as shown below in Table 1.

Signs of Alzheimer's or Other Dementias	Typical Age-related Changes
Memory loss that disrupts daily life, especially forgetting recently learned information. Forgetting important dates or events. Asking for the same information repeatedly. Over-reliance on memory aids.	Sometimes forgetting names or appointments but remembering them later.
Challenges in planning or solving problems. Trouble following familiar recipes, keeping track of bills or counting change.	Making occasional errors when balancing a checkbook.
Difficulty completing familiar tasks at home, at work or at leisure. Trouble driving to a familiar place, managing a budget at work or remembering the rules of a favourite game.	Occasionally needing help to use the settings on a microwave or record a television show.
Confusion with time or place. Losing track of date, seasons and the passage of time. Forgetting where they are or how they got there.	Getting confused about the day of the week but figuring it out later.
Trouble understanding visual images and spatial relationships. Difficulty reading, judging distance and determining colour or contrast.	Vision changes related to cataracts, glaucoma or age-related macular degeneration.
New problems with words in speaking or writing. Trouble following or joining a conversation. Struggling with vocabulary, problems finding the correct word or calling things by the wrong name.	Sometimes having trouble finding the right word.
Misplacing things and losing the ability to retrace steps. Putting things in unusual places and are unable to go back over steps to find them.	Misplacing things from time to time and retracing steps to find them.
Decreased or poor judgement. Changes in decision-making and may pay less attention to grooming themselves.	Making a bad decision occasionally.
Withdrawal from work or social activities. Trouble keeping up with favourite sports teams, remembering how to do a favourite hobby or being social.	Sometimes feeling weary of work, family and social obligations.
Changes in mood and personality. May become confused, suspicious, depressed, fearful or anxious. May be easily upset at home, work, with friends, or in places where they are out of their comfort zones.	Developing very specific ways of doing things and becoming irritable when a routine is disrupted.

Table 1: Signs of AD and Other Dementias Compared with Typical Age-Related Changes.

A comparison between typical age-changes in behaviour or cognitive ability with signs of AD and other dementias. ("2020 Alzheimer's disease facts and figures," 2020).

It is believed that AD onset begins up to 20 years before symptoms are first noticed and is classified into different stages, depending on the severity, ranging from mild cognitive impairment (MCI) to dementia (Figure 1). The length of each stage differs between individuals, which is most likely affected by age, genetics, gender, diet, and other factors ("2020 Alzheimer's disease facts and figures," 2020).

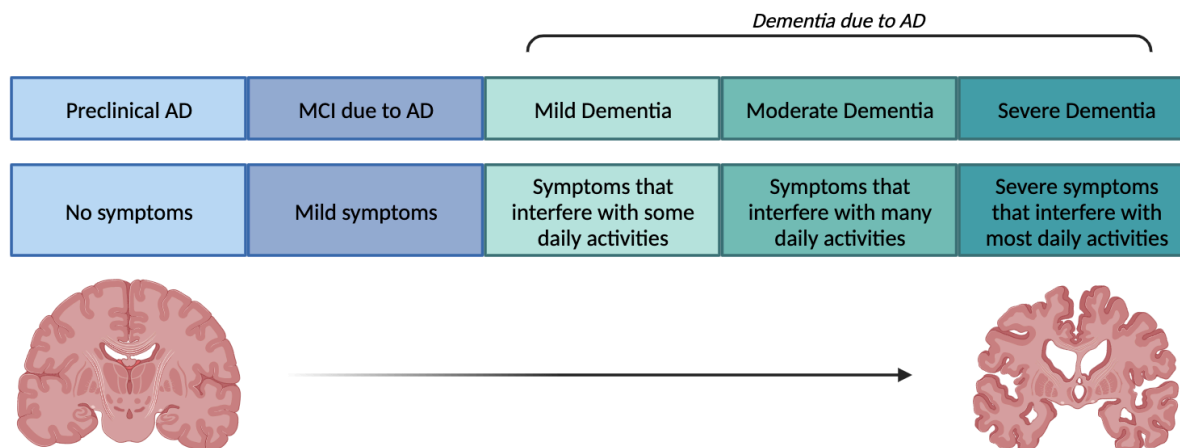


Figure 1: The Different Stages of Alzheimer's Disease

The different stages of are shown in three categories with descriptions of the severity of the accompanying symptoms for each stage. Image created by myself using BioRender.com.

In the preclinical phase of AD there are early physiological changes in the brain changes, such as biomarkers, but there are no noticeable symptoms of the disease, such as memory loss. The disease is only evident once it has significantly progressed, given symptoms only begin in the late stage of AD, which makes an early diagnosis extremely difficult. Measurable brain changes include increased levels of beta-amyloid, shown on positron emission tomography (PET) scans, and through analysis of cerebrospinal fluid (CSF) ("2020 Alzheimer's disease facts and figures," 2020).

Individuals with MCI due to AD have biomarker evidence of brain changes but will also exhibit subtle cognitive issues as the brain can no longer fully compensate for the damage to neuronal cells ("2020 Alzheimer's disease facts and figures," 2020). It has

been found that approximately one in four people, around 23.4%, with MCI will go on to develop AD (McGrattan et al., 2022).

Dementia is the last stage of AD and is split into three levels depending on the severity of the dementia which is ultimately a reflection on the degree of damage to nerve cells. Dementia as a result of AD is characterised by changes in memory, thinking, or behaviour which affects ability to function in daily life ("2020 Alzheimer's disease facts and figures," 2020).

In mild Alzheimer's dementia, most are able to function independently and are likely to only require assistance with some activities, while moderate Alzheimer's dementia includes difficulty with communication, completing routine tasks and personality and behavioural changes. In the last and most severe stage of Alzheimer's dementia, individuals will require assistance with activities of daily living and the effects of the disease on physical health are apparent ("2020 Alzheimer's disease facts and figures," 2020). Alzheimer's is ultimately fatal as the damage to nerve cells in the brain progresses with eventual damage to areas of the brain involved in movement, swallowing, and general physical functioning which leads to eventual death.

1.1.2 Pathology of Alzheimer's Disease

AD is pathologically characterised by accumulation of extracellular amyloid beta ($A\beta$) plaques, and intracellular neurofibrillary tangles (NFT) consisting of hyperphosphorylated Tau, a microtubule-associated protein (Feringa and van der Kant, 2021). Macroscopically, the disease is characterised by cerebral cortical thinning and atrophy, where synapse and neuronal loss contribute to the atrophy (Buccellato et al., 2021). Microscopically, identifiers of AD are neuritic amyloid plaques and NFTs (Buccellato et al., 2021).

The accumulation of these extracellular $A\beta$ plaques and intracellular abnormal tau is what is associated with the brain changes in AD. $A\beta$ plaques block communication

between neurons at synapses, which in turn affects the flow of information between the brain's neuronal circuits ("2020 Alzheimer's disease facts and figures," 2020). NFTs are responsible for disrupting the transport of nutrients and essential molecules inside neurons, contributing to neuronal damage and death.

Other AD associated pathological changes in the brain include inflammation. It is believed that the presence of amyloid beta plaques and NFTs activate microglia, immune system cells in the brain. This inflammation and microglia activation becomes problematic when it is chronic as this then causes damage to the brain.

The aetiology and pathogenesis in AD are not yet fully understood; which has thus far led to the failure in clinical trials of drugs targeting toward generally acknowledged molecules, especially A β (Guo et al., 2021). It is not known for sure if A β begins accumulating before abnormal tau, and increasing A β is what initiates the increase in tau or if there is a different sequence of events ("2020 Alzheimer's disease facts and figures," 2020). The fact that there is still no treatment that can effectively improve the crucial clinical outcomes of AD reveals that there is still a considerable gap in our knowledge of its complex pathogenesis, making a broader perspective urgent (Guo et al., 2021).

1.1.3 *A β Pathway in Alzheimer's Disease*

These A β plaques, also named senile plaques, are found in the extracellular space of the brain and are particularly present in the hippocampus region (Cheignon et al., 2018). These plaques are composed of aggregated A β , a 38-43 amino acid residue peptide, in the form of β -sheet rich fibrils. A β is generated after enzymatic cleavage by β and γ of Amyloid Precursor Protein (APP) (Figure 2; Cheignon et al., 2018).

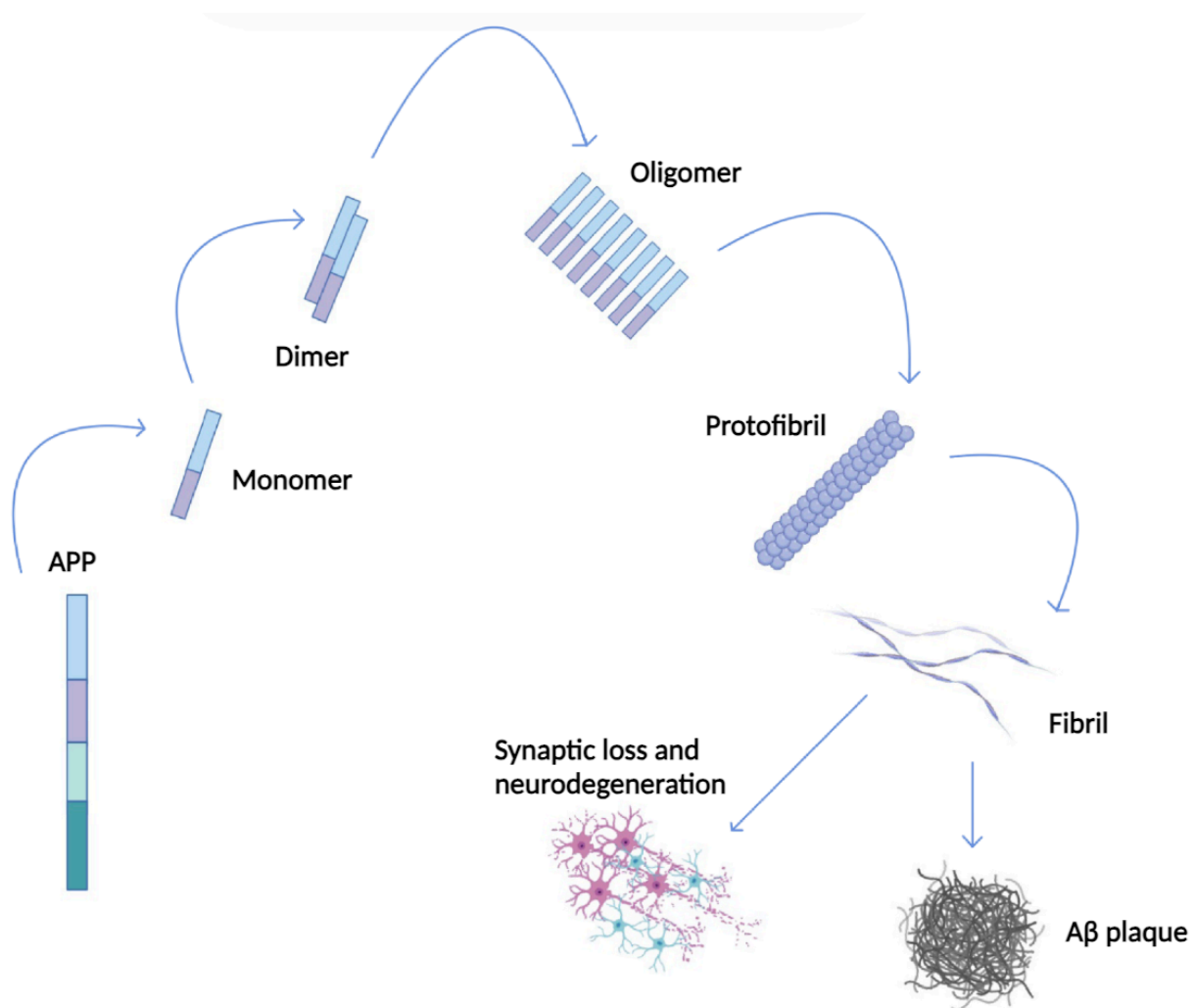


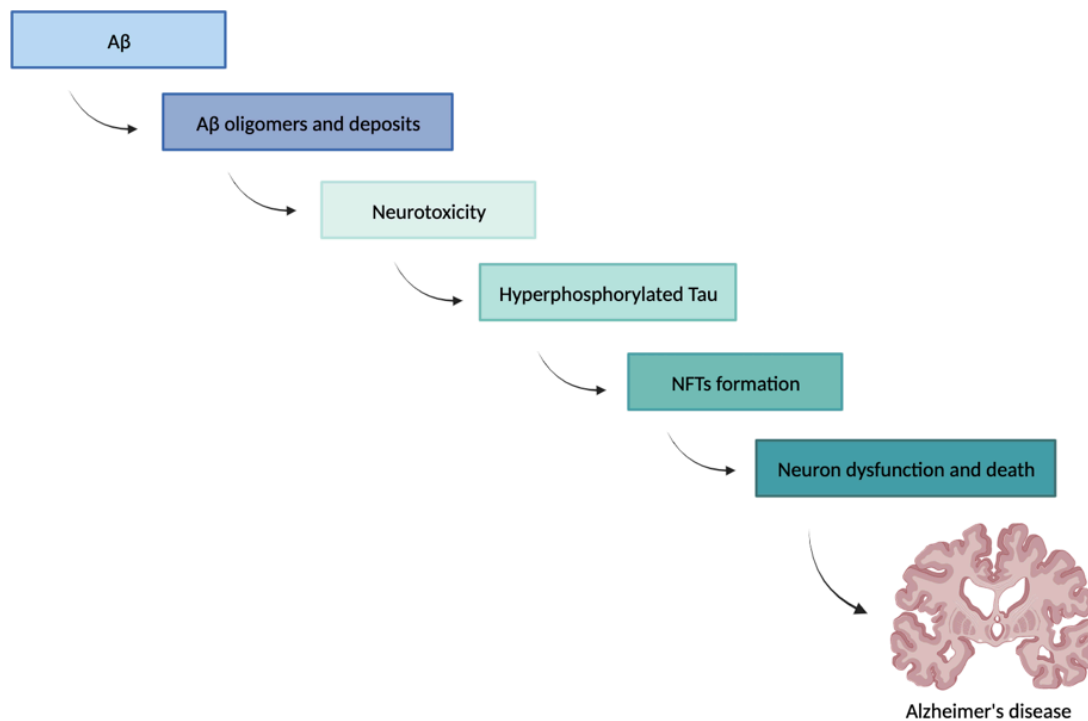
Figure 2: The Amyloid- β Aggregation Cycle.

Aggregation species of A β can exist in multiple forms such as monomers, dimer, oligomers, protofibril, fibril and amyloid plaques. These are found in a steady state where one form can convert to another in a bidirectional manner. These different A β species are characterised based on size, conformation state and solubility (with fibril and amyloid plaques being insoluble). Image created by myself using BioRender.com and information taken from Hampel et al., 2021. [Click here to enter text.](#)

This metabolism of APP occurs via two different pathways and is linked to the amyloid- β pathway in AD. These two pathways result in different by-products with different intrinsic functional properties, physiological roles, and pathophysiological implications (Hampel et al., 2021). The predominant pathway is non-amyloidogenic in which APP is initially cleaved by α -secretase and then by γ to form truncated A $\beta_{17-40/42}$ (P3) peptides

or by β -secretase to form the truncated $A\beta_{1-16}$ peptide (Figure 3; (Cheignon et al., 2018)). In the amyloidogenic pathway, which occurs to a minor extent, APP is cleaved consecutively by β - and γ - leading to the formation of full-length $A\beta$ peptides (mainly $A\beta_{1-40/42}$) (Figure 3; Cheignon et al., 2018). In both pathways, it is the cleavage by γ - that determines the length of the $A\beta$ peptide that is produced. $A\beta$ can be found in different aggregation species, such as monomers, dimers, oligomers, protofibril, fibril, and amyloid plaques where one form can convert to another bidirectionally (Figure 2; Hampel et al., 2021).

[A]



[B]

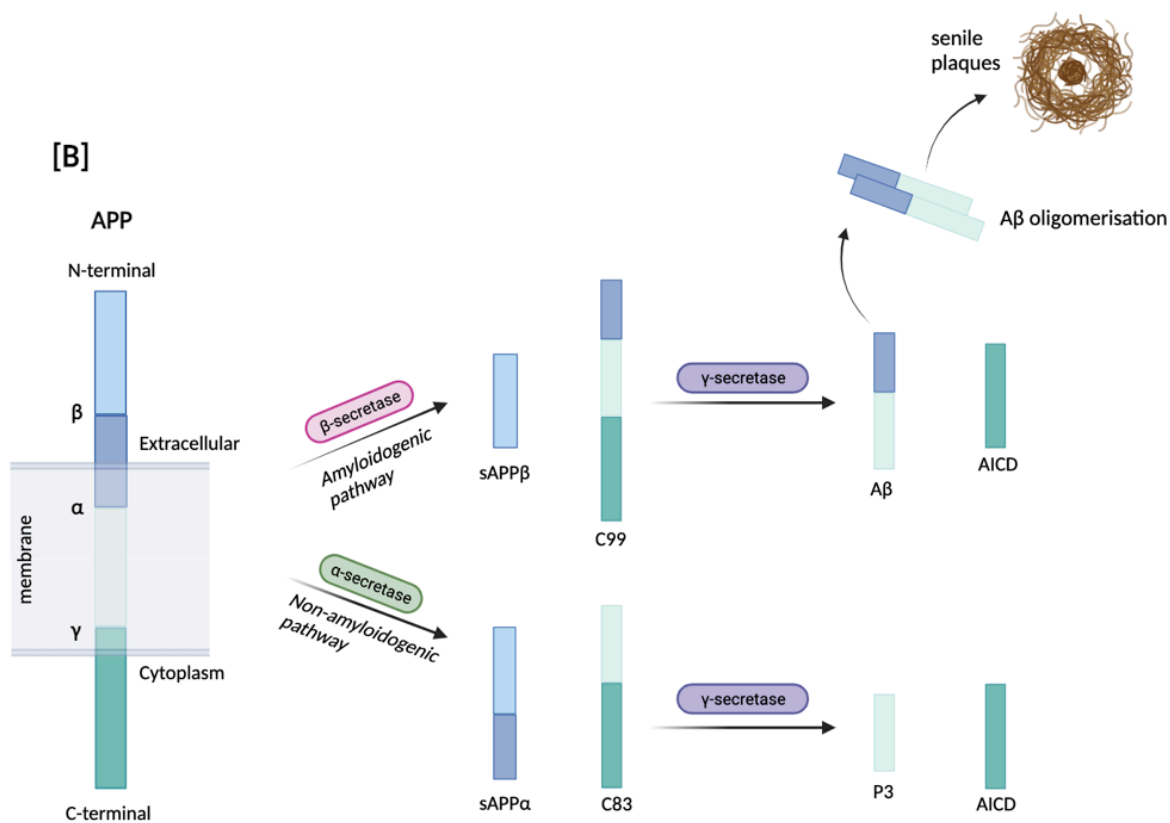


Figure 3: Mechanism of A β Toxicity and Processing of the Amyloid Precursor Protein (APP).

(A) Accumulating A β aggregates in the forms of fibrils and senile plaques. This aggregation alters kinase/phosphatase activity leading to Tau being hyperphosphorylated which then causes formation of NFTs and eventually synaptic and neuronal dysfunction. **(B)** The proteolytic processing of the amyloid precursor protein (APP) and amyloid- β genesis. APP is a transmembrane protein that is processed either via the non-amyloidogenic or amyloidogenic pathway. The non-amyloidogenic pathway prevents formation of A β as APP is first cleaved by α -secretase which generates soluble α -APP fragments and a C-terminal fragment α (CTF α , C83), C83 is then cleaved by γ -secretase which produces non-toxic P3 and generating APP intracellular domain (AICD) fragments. The amyloidogenic pathway is what generates A β . APP is first cleaved by β -secretase, producing soluble β -APP fragments (sAPP β) and C-terminal β fragment (CTF β , C99), and C99 is further cleaved by γ -secretase, AICD and A β . Image was created by myself using BioRender and data from Sun et al., 2015.

There are two major isoforms of A β , soluble A β_{40} and insoluble A β_{42} . A β_{42} is regarded as being more harmful as it is found in a higher percentage concentration in AD and is more prone to aggregate compared to A β_{40} (Sun et al., 2015). The presence of these two different isoforms is what led to proposal of the amyloid cascade hypothesis in the early 1990s (Cheignon et al., 2018). This hypothesis proposes that the inappropriate processing of A β precursor, and subsequent overproduction of A β_{42} , is what leads to an abnormal extracellular accumulation and aggregation of A β peptides in the brain, the formation of tau tangles, and ultimately neuronal death (Buccellato et al., 2021; Cheignon et al., 2018). These A β_{42} aggregates are described as the main constituent of neuritic plaques and are a hallmark of AD (Hampel et al., 2021). Hampel et al. (2021) further explain that the current and most accepted model indicates that A β pathophysiology is most likely an upstream pathophysiological event and may serve to initiate downstream molecular pathways such as tau misfolding, tau-mediated toxicity, tau tangles, and the spread of tau leading to cortical neurodegeneration (Hampel et al., 2021). This suggests that “AD is an A β -facilitated tauopathy leading to cognitive decline, MCI, and dementia” (Hampel et al., 2021).

1.1.4 Neuroinflammation and Glial Cell Activation

The interaction between A β and glial cells, the critical initiators of neuroinflammation, is a highly debated topic discussing whether neuroinflammation can initiate and sustain A β

dyshomeostasis, counteract it, or carry out both neuroinflammation and A β dyshomeostasis in a stage-dependent manner (Hampel et al., 2021). Most studies support neuroinflammation and glial cell activation as being key pathologic events in AD as these often accompany tangles and plaques in the cortex and, like NFTs, are seen to parallel clinical symptoms (Hampel et al., 2021; Busche and Hyman, 2020). Sustained A β elevation and aggregation can lead to a chronic response of the immune system whereby A β plaques interact with glial cells in the brain and trigger microglia “priming” (Busche and Hyman, 2020; Sun et al., 2015). This is significant as Busche and Hyman (2020) describes activated microglia as being one of the key pathophysiological features of AD.

Furthermore, neuroinflammation and neurodegeneration is promoted by these A β species (including oligomers, protofibrils, and fibrils) which stimulate the microglia to release pro-inflammatory cytokines and affect synthesis of anti-inflammatory cytokines (Busche and Hyman, 2020). The constant stimulation of microglia in this manner, can impair their ability to clear A β and neuronal debris and result in an increase in microglia-mediated neuronal death and loss of neuronal synapse, which is a factor contributing to AD pathogenesis (Sun et al., 2015).

1.2 Genetic Predisposition to Alzheimer's Disease

There are two major forms of AD which are sporadic or late onset Alzheimer's disease (LOAD) and the familial or early onset Alzheimer's disease (EOAD) (Cheignon et al., 2018). Less than 1 % of Alzheimer's cases are linked to the familial type of AD whilst over 95 % of AD patients have sporadic AD (Doifode et al., 2021).

Familial Alzheimer's, or EOAD, is characterised by autosomal dominance, the overproduction of A β , and often early-onset before the age of 65 (Doifode et al., 2021). Familial AD involves mutations of APP, PSEN1 and PSEN2, coding for APP, Presenilin 1 and Presenilin 2 (Presenilin 1 and Presenilin being subunits of γ -secretase)(Doifode et al., 2021). These have been identified as causative genes as they contribute to higher

A β production, with PSEN1 mutations specifically leading to an increase in A β ₁₋₄₂ (Feringa et al., 2021).

Sporadic Alzheimer's, or LOAD, is characterised by a later onset and is attributed to a combination of genetic and environmental factors, such as inheritance of the ϵ 4 allele of the apolipoprotein E (ApoE ϵ 4) gene, increasing age, and family history (Doifode et al., 2021). The ApoE ϵ 4 gene is regarded as the most common genetic risk for LOAD and is involved in A β clearance (Feringa and van der Kant, 2021). Homozygosity for the ApoE ϵ 4 allele has been shown to increase the risk of developing sporadic AD by 3-to 15-fold (Hampel et al., 2021). ApoE has three major variants; ApoE ϵ 2, ApoE ϵ 3, and ApoE ϵ 4 with the ApoE ϵ 3 allele being most common and ApoE ϵ 2 allele being least common (Hampel et al., 2021). ApoE is expressed in astrocytes and microglia and the different isoforms modulate various brain intracellular signalling pathways (Hampel et al., 2021). In AD, ApoE ϵ 4 is associated with increased aggregation of A β , formation of neurotoxic A β species, and plaque accumulation (Hampel et al., 2021). The ApoE ϵ 2 allele is associated with decreased risk of AD-related neurodegeneration compared to those with ApoE ϵ 3 homozygotes and ApoE ϵ 4 carriers (Hampel et al., 2021). In LOAD, there are also factors outside of genetics, such as diet, neuroinflammation, head trauma, diabetes, and high cholesterol that potentially are involved in AD aetiology (Doifode et al., 2021).

The imbalance between A β production and effective extracellular clearance of A β is regarded as an upstream event of A β dyshomeostasis and is associated with the protein misfolding, aggregation and extracellular accumulation in plaques which is observed in AD (Hampel et al., 2021). In familial AD (FAD) this imbalance is attributed to genetic-driven dysregulation of the amyloidogenic pathway, leading to overproduction of A β (Hampel et al., 2021). However, in the case of sporadic AD (SAD), it is attributed to failure of proteostasis networks and insufficient cerebral A β clearance (Hampel et al., 2021).

1.3 Oxidative Stress in Alzheimer's Disease

Redox stress (RS) is defined as an imbalance between the levels of reactive oxygen species (ROS) and/or reactive nitrogen species and antioxidant defences which can lead to oxidative damage to cells, molecules and biological systems (Buccellato et al., 2021). Due to the brain's high oxygen consumption (~20 % of the total body consumption) and relative low levels of antioxidant enzymes present, in comparison to other organs, it is highly susceptible to oxidative stress (Haddadi et al., 2014).

Oxidative stress (OS) is one of the main pathological features of AD, indicated by the presence of oxidative stress markers in neurofibrillary tangles and senile plaques in the Alzheimer's brain (Karapetyan et al., 2022). Buccellato et al. (2021) describes how the reaction of ROS with different substrates can result in protein, nucleic acid oxidation or lipid peroxidation which is noted as being an early change in the AD brain and is what led to the oxidative stress hypothesis in AD pathogenesis (Buccellato et al., 2021). To support this is the association of elevated A β ₁₋₄₀ and A β ₁₋₄₂ levels and increased levels of oxidation products from proteins, lipids, and nucleic acids in the hippocampus and cortex of AD patients (Cheignon et al., 2018).

Oxidative damage in the brain is involved in the molecular mechanisms which link accumulation of A β plaques and NFTs to microglia activation (Buccellato et al., 2021). It is suggested that oxidative stress is involved in the insufficient clearance of A β from the brain where ROS molecules have been associated with enhanced A β generation, misfolding, and aggregation (Thapa and Carroll, 2017). The tau protein, as seen in neurofibrillary tangles, is also linked to oxidative stress in AD. Oxidative stress is seen to induce modifications of the tau protein which, given its involvement in neuronal genomic DNA and cytoplasmic and nuclear RNA as a microtubule protein, would lead to increased DNA and RNA oxidation (Cheignon et al., 2018).

Subsequently, oxidative stress is one of the main factors in neuronal death in many neurodegenerative diseases (Haddadi et al., 2014). ROS are known to accelerate ageing and cause damage to many cells in the body, including neurons (Elfawy and

Das, 2019). Although, oxidative stress is associated with several neurodegenerative diseases, it is not established whether this OS is the cause or the consequence of pathology and is question that has yet to be answered. This question is of importance as it can possibly provide a therapeutic target should the point at which oxidative stress occurs in AD aetiology be known (Cheignon et al., 2018).

1.3.1 Microglial Activation in Alzheimer's Disease

Microglia are suspected to play a significant role not only in early AD pathogenesis but also in the progression of the disease, as reactive microglia around amyloid- β plaques and near to the tau protein in NFTs are a prominent characteristic of AD (Buccellato et al., 2021). These active microglia respond to damaged neuronal cells in a neuroprotective manner however this can quickly become a contributing factor to neuroinflammation and neurodegeneration (Tejera and T. Heneka, 2016; Sarlus and Heneka, 2017; Buccellato et al., 2021). This is because microglia respond to A β , or other damage-associated molecules released by injured cells, by producing reactive oxygen species and releasing pro-inflammatory cytokines and chemokines (Buccellato et al., 2021). This can quickly lead to oxidative stress in the brain whereby there is an overproduction of ROS and a decrease in antioxidant defences (Buccellato et al., 2021). Moreover, increased levels of chemokines and cytokines are noticed very early on in AD pathogenesis, even before clinical onset, which demonstrates their role in the progression of the disease (Buccellato et al., 2021).

1.3.2 Mitochondrial Dysfunction in Alzheimer's Disease

The diagnosis of morphologically and functionally impaired mitochondria, which are prone to excessive formation of ROS, is one of the earliest pathological changes that can be seen in AD (Elfawy and Das, 2019; Buccellato et al., 2021). These mitochondria are considered one of the sources of ROS and also the target of oxidative stress since mitochondrial DNA (mtDNA) is especially susceptible to oxidative damage (Buccellato et al., 2021). These mitochondrial abnormalities are observed in neuronal cells without

NFTs which could suggest that impaired mitochondrial function is one of the earliest pathological changes in AD (Buccellato et al., 2021)

Studies on oxidative damage to mitochondria have led to the mitochondrial-cascade hypothesis in AD pathogenesis (Buccellato et al., 2021). This hypothesis proposes that effective mitochondrial functioning declines during ageing which contributes to elevated production of ROS (Buccellato et al., 2021). Elevated levels of ROS species in the brain, as a result of impaired mitochondria, can lead to further oxidative stress which causes damage to the brain via mechanisms such as elevated Ca^{2+} and release of excitatory amino acids (excitotoxicity), all resulting in enhanced neurotoxicity (Elfawy and Das, 2019).

1.3.3 Markers of Oxidative Stress in Alzheimer's Disease

Oxidative damage products deriving from oxidation of lipids, DNA, RNA, or advanced glycation end products can be used as biomarkers for neurodegeneration and AD (Buccellato et al., 2021). This is because oxidative stress markers and indicators of oxidative damage, such as lipid peroxidation (LP) and protein carbonyls, in neurons in the brain can indicate neuronal death which is commonly associated with AD.

Neuronal cells generate significant ROS and oxidative stress which is seen in neurons in the form of lipid peroxidation, one of the pathological markers found in AD brain tissue (Thapa and Carroll, 2017). Lipid peroxidation is responsible for the damage that occurs to the membranes of neurons and occurs because of the high number of oxidisable polysaturated fatty acids in the brain (Thapa and Carroll, 2017). Evidence of significant lipid peroxidation in the brain can therefore be considered an indicator of AD pathogenesis.

Additionally, several protective mechanisms, such as antioxidant enzymes, are generated in response to oxidative stress and are reported to be a relatively early event in AD pathogenesis (Thapa and Carroll, 2017). These antioxidant enzymes are responsible for degrading ROS, mediating redox reactions, and decreasing oxidative

stress (Thapa and Carroll, 2017). Examples of these antioxidant enzymes are superoxide dismutase (SOD), glutathione reductase, and catalase which have all been identified in damaged neurons in AD (Buccellato et al., 2021).

Specifically, the presence of SOD in A β plaques is a reliable indicator of oxidative stress in AD pathology as reduction of SOD has been demonstrated post-mortem in mitochondrial and synaptosomal fractions in the frontal cortex of AD patients (Buccellato et al., 2021; Thapa and Carroll, 2017). Thapa and Carroll also used serum analyses of AD patients to illustrate the weakened antioxidant defence mechanisms, characteristic of AD displaying a decreased level of SOD.

A distinctive pathological feature of AD is neuronal death and synapse loss which is, in part, the result of enhanced oxidative stress. Caspase-3 is a key mediator in apoptosis and can be used to identify the extent of cell death in the brain. Activated Caspase-3 in the neurons of AD brains, near to NFTs and A β plaques supports the notion of synaptic and neuronal degeneration during AD pathogenesis (Khan et al., 2015). Caspase-3 also plays a role in the cleaving of APP which generates A β isoforms (Khan et al., 2015). Therefore, Caspase-3 is an additional protein marker which can be used to describe AD pathology. Proof of apoptosis, through detection of Caspase-3 in neuronal cells, is an additional method to detect oxidative stress, neuronal death, and synapse loss in the AD brain.

1.3.4 Caspase-3 in the Alzheimer's Disease Brain

Caspases form a unique class of cysteine aspartate-specific proteases that present key signalling of apoptosis (Khan et al., 2015). Activated Caspase-3 is of specific interest as has been identified as a key mediator of neuronal apoptosis in AD where it has been detected in neurons and is often localised near to neurofibrillary tangles (NFTs) and senile plaques (Khan et al., 2015). Activated Caspase-3 plays a role in the cleaving of APP, which generates A β -containing peptides, and has been implicated in contributing to synaptic degeneration during AD progression (Khan et al., 2015).

1.4 The Gut-Brain Axis

Over recent years, the gut microbiome (GM) has been implicated in many neurodegenerative diseases, specifically AD. The gut microbiome is an all-encompassing term for the microorganisms, and all their genetic materials, found in the gastrointestinal (GI) tract and is known to play an important role in keeping homeostasis and modulating functions for most body systems, such as the central nervous system (CNS) (Guo et al., 2021). This connection between the GM and AD was created when the link between the gut and brain, referred to as the gut-brain axis (GBA), was discovered. The GBA is a bidirectional communication pathway that exists between the GM and the brain, in which the CNS is regulated by the GI microbiota through the vagus nerve and neural, immune, endocrine, and metabolic signalling (Figure 4; Doifode et al., 2021).

GM dysbiosis facilitates secretion of amyloid and lipopolysaccharides (LPS) which contribute to AD pathogenesis given that GI permeability and blood-brain barrier (BBB) permeability increases during ageing, thereby decreasing the brain's protective barrier (Kesika et al., 2021).

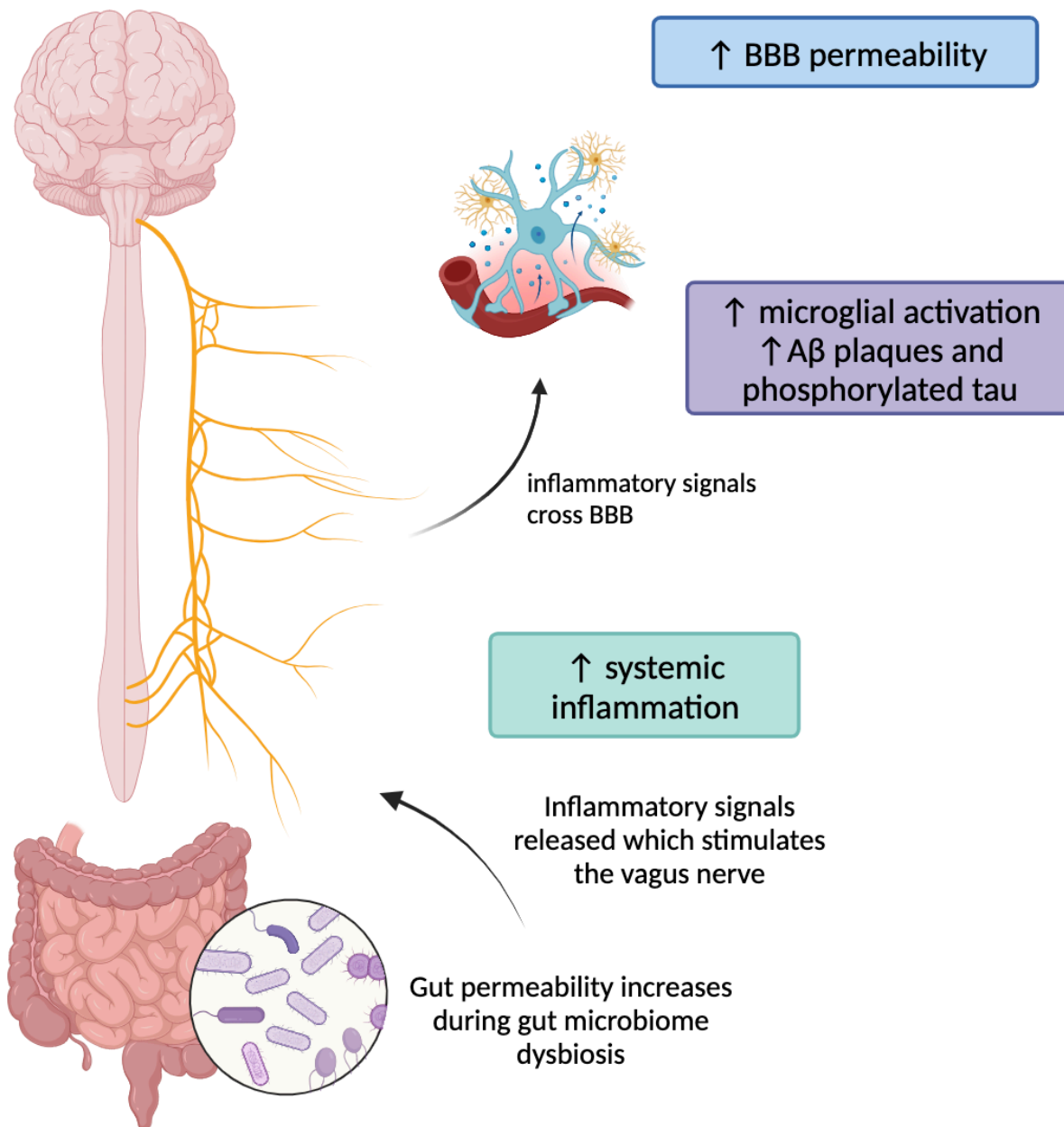


Figure 4: The Interplay Between Enteric Dysbiosis and Alzheimer's Pathology.

The immune cells present in the gastrointestinal (GI) tract can release inflammatory signals during GM dysbiosis. These inflammatory signals have been seen to be able to cross the blood brain barrier (BBB) where they have then been seen to activate glial cells and the neuroinflammatory pathway. Imaged created by myself using BioRender.com and information from Doifode et al., 2021.

Amyloids are secreted by some of the microbes present in the human GM and while these do not have identical amino acid sequences to human A β ₄₂, they still have similar pathogen-associated molecular patterns (PAMPs) and have been proven to promote A β aggregation (Guo et al., 2021). The release of LPS has been shown to cause a leaky BBB with increased blood to brain influx and decreased brain to blood efflux which promotes A β accumulation and neuroinflammation (Kesika et al., 2021; Figure 4).

Additionally, ageing has been seen to induce changes in the gut microbial composition with increased pro-inflammatory bacteria and low abundance of anti-inflammatory bacteria (Kesika et al., 2021). GM dysbiosis, that results from differences in bacterial composition and alterations in metabolite production, can lead to mitochondrial dysfunction which is an accepted early event of AD (Guo et al., 2021). This can have a consequential reaction in that oxidative stress and the inflammatory response are increased (Guo et al., 2021).

1.5 Cholesterol and Alzheimer's disease

Levels of cholesterol in the brain are tightly regulated in order to maintain regular physiological functioning, however there is increasing evidence that suggests excessive cholesterol accumulates in the brain in AD and is associated with AD pathogenesis (Feringa and van der Kant, 2021). This is further shown by the accumulation of cholesterol in A β plaques in AD patients as well as the existence of a strong positive correlation between the levels of cholesterol in the brain and the severity of dementia in AD patients (Feringa and van der Kant, 2021).

The brain is dependent on endogenous cholesterol synthesis, which is thought to take place mainly in astrocytes where cholesterol is transported via apolipoproteins to neurons (Trindade de Paula et al., 2016; Rivera et al., 2019; Feringa and van der Kant, 2021). These neurons rely on the transport of cholesterol, and cholesterol precursors, from astrocytes however recent studies have provided evidence of lipids being transported in the opposite direction, from neurons to astrocytes (Feringa and van der Kant, 2021). This type of transport occurs during high levels of oxidative stress where

neuronal lipids will become peroxidized and will then be transported to astrocytes as the neurons are not able to manage the peroxidized lipids (Feringa and van der Kant, 2021).

The blood-brain barrier is responsible for regulating cholesterol levels in the brain by keeping cholesterol metabolism in the CNS separate from the rest of the body, such that diet-derived cholesterol does not enter the brain (Feringa and van der Kant, 2021). During ageing, and the result of GM dysbiosis, the BBB can lose its integrity thereby allowing in peripheral cholesterol which effects brain cholesterol levels. Additionally, neurodegeneration itself effects cholesterol levels in the brain as dying neurons produce increased amounts of debris that is rich in cholesterol (Feringa and van der Kant, 2021). This excess cholesterol in the brain cannot be fully managed by astrocytes and microglia, which then induces an inflammatory response in the brain (Feringa and van der Kant, 2021). These concepts relating to excess cholesterol in the brain, as a result of an impaired BBB, are important as they provide a link between a high-fat diet and AD pathogenesis.

Cholesterol also affects APP processing and A β generation in neurons. The cholesterol present in cell membranes can bind to APP which promotes its insertion into the phospholipid monolayers, thereby promoting β -secretase activity and subsequent formation of A β_{1-42} by favouring the amyloidogenic pathway (Cheignon et al., 2018). This applies, more specifically, to esterified cholesterol which enhances A β formation; making esterified (not free) cholesterol and free cholesterol the modulator of amyloidogenesis (Figure 5; Cheignon et al., 2018).

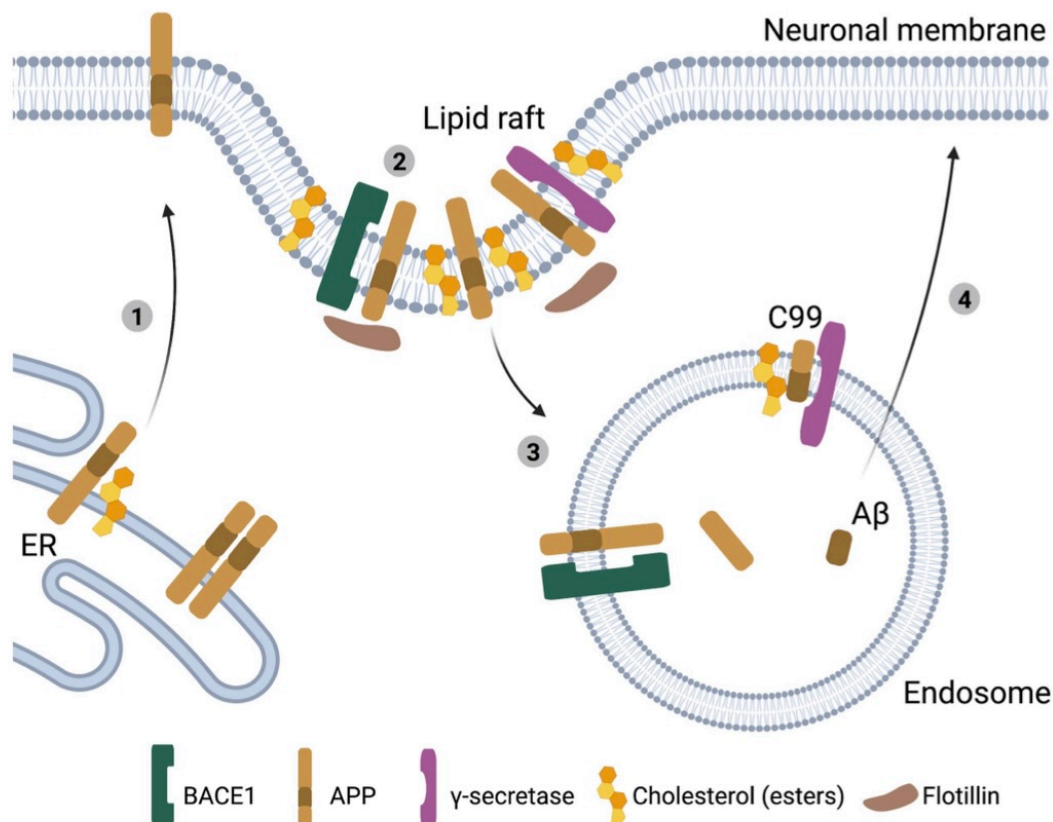


Figure 5: The Interaction Between APP Processing and Cholesterol in Neurons.

(1) High endoplasmic reticulum-cholesterol (ER) levels prevent APP dimerization and its transport to the plasma membrane. (2) Cholesterol or cholesterol-esters in the plasma membrane stimulate APP, BACE1 and γ-secretases to cluster in lipid rafts. (3) Lipid rafts undergo clathrin-mediated endocytosis which results in amyloidogenic processing of APP to Aβ (Feringa and van der Kant, 2021).

The role of cholesterol in AD pathogenesis is particularly relevant for LOAD as the genetic risk factors of ApoE ε4 are functionally linked to cholesterol metabolism (Feringa and van der Kant, 2021). Apolipoprotein acts as a cholesterol and lipid character and is crucial for transport of cholesterol precursors from astrocytes to neurons (Feringa and van der Kant, 2021). The exact mechanism by which ApoE affects cholesterol in the brain is not fully understood, but dysregulation in lipid metabolic genes in ApoE ε4 astrocytes have been observed in AD brain samples. This implies that ApoE ε4 plays a role in the regulation of cholesterol levels in the AD brain.

1.6 *Drosophila melanogaster*: A Model Organism

1.6.1 *Drosophila melanogaster* Genetics

Drosophila melanogaster, commonly known as a fruit fly, is a useful genetic model system that has been used for over 100 years to study human disease (Bulus et al., 2020). As *Drosophila* are relatively inexpensive to maintain, reproduce rapidly and have extensive homology with human genetics, they are an ideal model to investigate the cellular, molecular, physiological and behavioural traits of human disease, such as AD (Figure 6; Bulus et al., 2020).

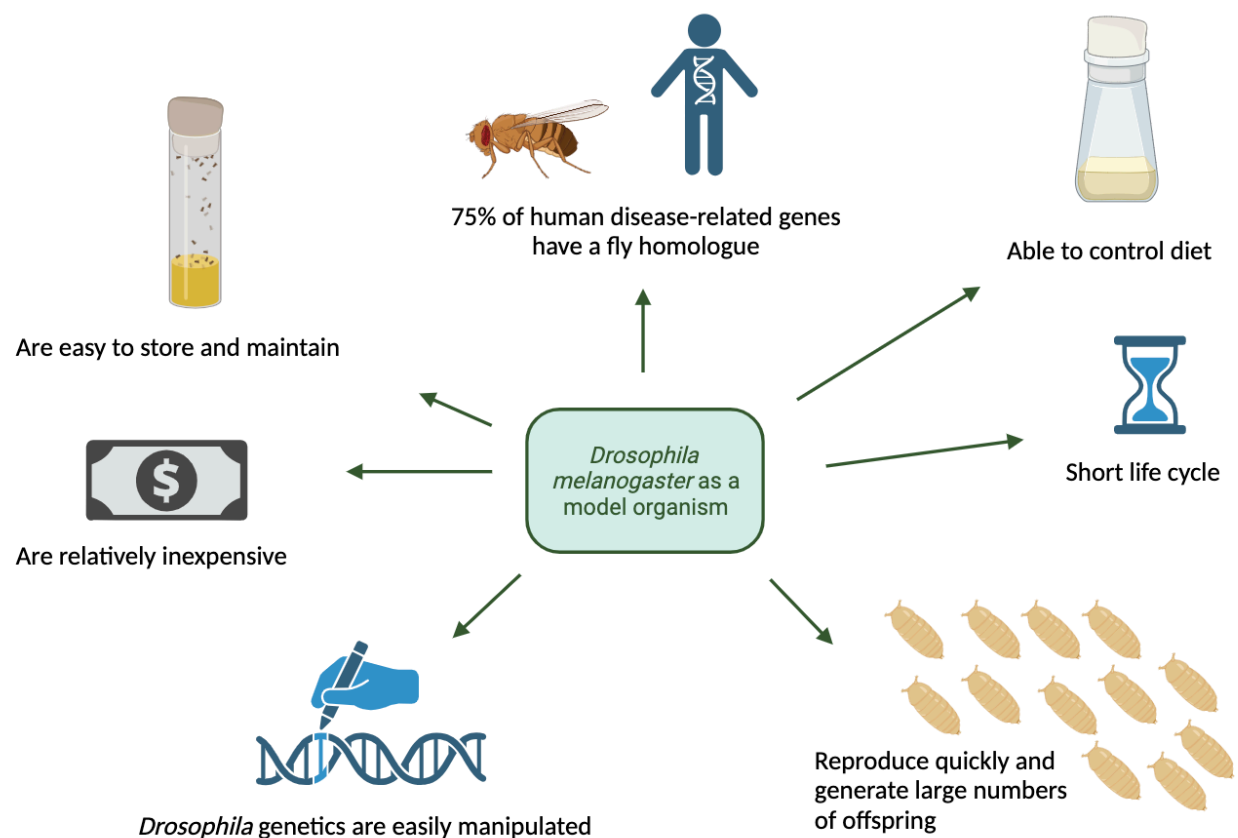


Figure 6: *Drosophila melanogaster* as a Model Organism for Research of Human Disease

Advantages of *Drosophila melanogaster* as a model organism. *Drosophila* are easy to breed and maintain in large numbers, easy to manipulate, take up little space, have a short life cycle with abundant offspring and about 75 % of human disease-related genes have fly homologs. Image created myself using BioRender.com.

In the Alzheimer's model used in this work, *Drosophila* express genes representative of familial and sporadic Alzheimer's (A β arc and hApoE4). Expression is driven using the GAL4 driver in the UAS- GAL4 system (Figure 7). This driver line contains a specific tissue promoter (elav) which drives the expression of the yeast GAL4 protein in neurons (Anoar et al., 2021). The UAS line, the responder line, is what carries the gene of interest which is expressed downstream of the promoter (Anoar et al., 2021). This driver line is then crossed with the responder line, which causes the GAL4 driver and the UAS-transgene to come together (Anoar et al., 2021).

To perform genetic crosses in this system, it is vital that male and female flies that carry the appropriate genotypes are carefully selected. For example, selected GAL4x females must be virgin flies (selected before they are randomly fertilised by males in their vial of origin), and young male flies that are selected must contain the UAS construct.

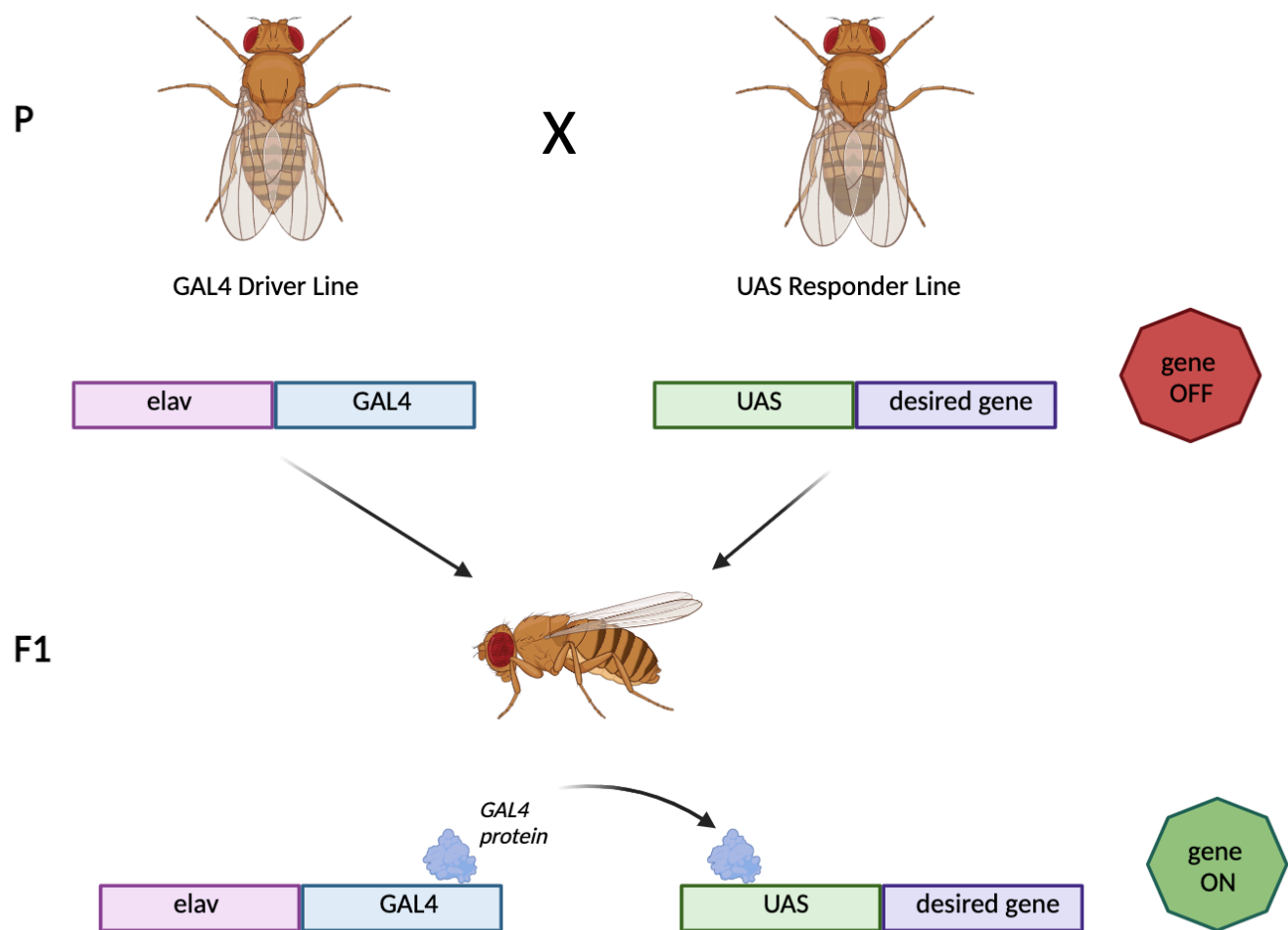


Figure 7: The UAS-GAL4 System in *Drosophila melanogaster*.

The GAL4 driver line contains a tissue specific promoter which drives the expression of yeast GAL4 protein in neurons. The responder line carries the gene of interest cloned down-stream of the UAS promoter, this, however, is not expressed. When these two lines are crossed, the GAL4 driver and UAS-transgene construct will come together in one animal. The GAL4 will be expressed in neurons, bind the UAS promoter and drive the expression of the gene of interest specifically in neurons. Image created by myself using BioRender.com. [Click here to enter text.](#)

1.6.2 *Drosophila melanogaster* as an Alzheimer's Disease Model

Drosophila melanogaster are an excellent model to study the biology of ageing and age-related functional impairments because of its short lifespan and many age-related functional senescence similarities with human subjects (Haddadi et al., 2014). The *Drosophila* brain also has a similar but more simple central nervous system compared

to humans, both consisting of a protective (blood/haemolymph) brain barrier (Schirmeier and Klämbt, 2015) as well as neurons and supporting glia using the same neurotransmitters (Hui Ping Tan and Azzam, 2017). Cellular processes and age-dependent behaviours in human neurodegeneration, such as oxidative stress, A β production, neuronal and locomotor ability, are similarly exhibited in *Drosophila* (Hui Ping Tan and Azzam, 2017).

Creating an AD model in *Drosophila* involves use of the UAS-GAL4 system to induce expression of genes representative of familial and sporadic Alzheimer's, UAS-A β arc and UAS-hApoE ϵ 4. Use of the UAS-hApoE ϵ 4 gene (hApoE4) allows overexpression of the human ApoE ϵ 4 allele in the *Drosophila* brain which is the greatest genetic risk for sporadic, late-onset AD (Hui Ping Tan and Azzam, 2017). This model "exhibits progressive neurodegeneration, memory impairment, and early death" (Hui Ping Tan and Azzam, 2017). Use of the UAS-A β arc gene (A β arc) allows for an A β toxicity model in which there is abnormal accumulation of A β in the brain. These flies exhibit shortened life span, climbing defects, and neurodegeneration phenotypes which makes them a suitable model for familial, early-onset AD (Anoar et al., 2021).

Additionally, *Drosophila* can be used in multiple assays to assess neurodegeneration, which provides different methods to observe the effects of the disease process (Bulus et al., 2020). Examples of these methods include lifespan analysis, climbing assays, histological staining of the brain as well as assessment of the neuromuscular junction. These assays allow for physiological, cellular and molecular changes, as well as functional impairments, involved in ageing and neurodegeneration to be observed.

There are some limitations to using a *Drosophila* AD model in that the APP gene in humans is not as well conserved in *Drosophila* as some genes are for other human diseases. APPL is the gene coding for A β in *Drosophila* and is an orthologue, but not identical, to the human APP gene which codes for A β in humans (Bulus et al., 2020). The region specific to A β protein coding is not thoroughly conserved between *Drosophila* and humans, however it is similar enough in function and in disease to

impact the *Drosophila* mutants and are therefore a useful baseline model to investigate the effect of A β on disease (Bulus et al., 2020). There are other reported *Drosophila* genetic lines used to study neurodegeneration, such as triose phosphate isomerase, that are better conserved, which gives a better comparison to humans, however these don't target the A β protein specifically which was the aim of this work.

Additionally, BACE1 is an important enzyme in APP processing in AD and is regarded as a prime drug target for therapeutic treatment of AD. *Drosophila* have a homologue, called dBACE, for the human BACE enzyme which further exhibits why they may serve as a good model for AD (Bolkan et al., 2013).

All conclusions and observations from this AD model would need confirming in a mammalian model in a manner that would replicate this study. This project was a preliminary study in which flies are useful because of the reasons listed in section 1.6.1 above. Therefore, while this *Drosophila* AD model is not completely identical to AD in humans, it provides a similar baseline from which to draw conclusions and develop further research.

1.7 Aims and Hypothesis

Drosophila melanogaster is an ideal organism to model human disease, such as AD. Although the physiological and pathological symptoms of AD in *Drosophila* are not identical to that in humans, it still provides a suitable model to investigate the effects of genetic and environmental factors on AD aetiology.

The aim of this work was to determine whether a high-fat diet contributes to AD pathogenesis and onset of the disease. An additional aim was to determine if there is a link between genetic risk for AD and environmental factors such as a high-fat diet. It was hypothesised that a high-fat diet will affect AD symptoms and pathogenesis in *Drosophila* and that this effect will be most significant in the A β arc expressing flies, then hApoE4 expressing flies, and have less of an effect on the W₁₁₁₈ flies. Furthermore, a

gender difference was investigated as AD pathologies have been reported to differ in males and females.

2 Methods:

2.1 Drosophila melanogaster Stocks and Genetics

Drosophila melanogaster in the NF experimental group were reared on a standard cornmeal-agar media, without active yeast, made up of water, cornmeal, agar, soya flour, yeast, sugar syrup, propionic acid and nipagin. *Drosophila melanogaster* in the HF experimental group were reared on the same media in which virgin coconut oil (10 %) was added. The composition of the NF and HF diets is outlined below in Table 2.

	NF	10% HF
Energy (kcal/g)	4.039	4.49
Carbohydrate (weight, %)	89.26	80.13
Protein (weight, %)	8.66	7.77
Total fat (weight, %)	2.07	12.08
Total saturated fat (weight, %)	1.08	10.00
6:0	0	0.03
8:0	0	0.05
10:0	0	0.49
12:0	0.03	0.61
14:0	0.13	2.02
16:0	0.61	1.71
18:0	0.20	0.61
20:0	0.01	0.01
24:0	0.001	0.002
Total monounsaturated fatty acids (weight, %)	0.60	1.35
14:1	0.007	0.01
16:1	0.02	0.04
18:1	0.55	1.30
Total polyunsaturated fatty acids (weight, %)	0.38	0.72
18:2 n-6	0.40	0.63
18:3 n-6	0.01	0.02
18:1t	0.04	0.06
18:2t	0.005	0.007

Table 2: Composition of Regular Diet (RD or NF) and 10 % High-fat Diet (HFD or HF).

Table created by myself with data taken from (Trindade de Paula et al., 2016).

All *Drosophila* were kept in an 18 °C incubator (Sanyo) with a controlled 12 h light/12 h dark cycle, in standard vials. This temperature ensured the HF food remained solid and the flies did not get stuck. While Trindade et al., performed their experiments at 25 °C, and were able to keep the flies alive, a prior masters student had attempted to keep the flies at 25 °C in HF food and was unsuccessful. It was found that the coconut oil separated from the fly food and the flies quickly perished after they got stuck or drowned in the oil. This past masters student attempted to contact the authors of the Trindade et al. article, to determine how they successfully kept the flies in a HF diet at 25 °C, but received no response. Therefore, in order to conduct this experiment, it was necessary to keep the flies at 18 °C.

A full list of *Drosophila* stocks is shown below (Table 3). Each genotype was kept separate in plastic vials (Greiner Bio-One, Stonehouse, UK) and vials were changed once a week. Acute CO₂ exposure was used to anesthetise the flies, which were then transferred to a porous fly pad (Genesee Scientific, California, USA) to achieve desired, consistent diffusion of CO₂.

To consistently achieve the expected progeny phenotype, genetic crosses were performed using virgin females and males of the required genotype. Virgin females were identified under a dissecting microscope (Zeiss, Germany) based on closed wings and the presence of the meconium, a dark spot in the abdomen, and were collected within 8 hours of hatching. Crosses were flipped every 2-3 days to generate multiple progeny-yielding vials.

Stock	Source
W ₁₁₁₈	BDSC
GAL4 (X)	BDSC
UAS-hApoE ϵ 4	BDSC
UAS-A β arc	Dr Nathan Woodling, UCL

Table 3: A List of Drosophila Lines Used in this Project.

Abbreviation: BDSC, Bloomington Drosophila Stock Centre (Bloomington, USA).

Given that this was a preliminary study, the relevant genotypes were not backcrossed into a standardised genetic background for at least six generations. Future studies within this lab will address this.

2.2 General Methods

2.2.1 Experimental Set Up

A *Drosophila melanogaster* Alzheimer's model was used to investigate the effect of a high-fat diet on AD pathophysiology, involving the gut-brain axis. Wild type flies (W₁₁₁₈) were used as a control while the A β arc- and hApoE4-expressing flies were used to represent familial AD and sporadic AD models respectively. For this thesis, the terms A β arc and A β ₄₂ and hApoE4 and ApoE4 were used interchangeably when referring to the specific fly lines.

Flies were separated by sex upon hatching and were fed a normal diet until 5 days of age where they were then separated into the two diet groups, HF and NF. The flies in the two groups, HF and NF, were then further separated into the two experimental age groups, 10d and 45d. The flies in the NF group received a standard cornmeal-agar food

while the HF group received a high-fat food that was created using standard cornmeal-agar food into which virgin coconut oil was added (10 %).

This method of grouping the flies, by sex, diet, and age, was done for all three genotypes (W_{1118} , $A\beta_{arc}$ and hApoE4). Flies were collected following the additional 5 days and 40 days on their respective HF or NF diets (not including the initial 5 days spent on a NF diet for all experimental groups). For each age group a 2-3 day window was allowed, meaning flies were between 10-13 days old (10d) or 45-48 days old (45d). The two different age groups were created with the intention of comparison between young and old. Therefore, this experimental setup yielded a total of 24 experimental groups which is summarised below in Table 4.

W1118	Male	NF	10d
			45d
		HF	10d
			45d
	Female	NF	10d
			45d
		HF	10d
			45d
UAS-A β arc	Male	NF	10d
			45d
		HF	10d
			45d
	Female	NF	10d
			45d
		HF	10d
			45d
UAS-ApoE ϵ 4	Male	NF	10d
			45d
		HF	10d
			45d
	Female	NF	10d
			45d
		HF	10d
			45d

Table 4: Summary of Different Experimental Conditions.

The experimental conditions consisted of three different genotypes. Flies from each respective genotype were separated further which yielded a total of 24 experimental groups, divided according to genotype, sex, diet, and age.

At either 10 days or 45 days of age, the flies were collected and used in different assays to characterise signs of neurodegeneration and AD pathogenesis. These assays included behavioural assays, such as a climbing assay, and a longevity assay. These assays were done to test mobility (negative geotaxis) and survival, respectively. Assays to test for markers of oxidative stress were performed, such as ELISAs, measuring SOD activity and lipid peroxidation (LP). Additionally, brains were dissected and immunostained to detect the presence of cleaved Caspase-3 in the brain.

2.3 Biochemical Assays

To determine if there was evidence of oxidative stress in the *Drosophila* brain, two different biochemical assays were conducted to measure SOD activity and levels of LP. A Bradford assay was run (Thermo-Scientific Pierce 660 nm Protein Assay) prior to each biochemical assay to normalise data for total protein concentration in each sample.

A SOD Assay (Sigma-Aldrich Superoxide Dismutase Activity Assay Kit) was performed to measure the enzymatic activity of SOD. Activity is determined by measuring the decrease in superoxide anions which are generated by the enzyme xanthine oxidase (XO). The kit measures the SOD activity in terms of percent inhibition or in units of activity.

Additionally, a Lipid Peroxidation (MDA) Assay (Sigma-Aldrich Lipid Peroxidation (MDA) Assay Kit) was performed to measure the amount of oxidative damage in the samples. The assay works by measuring the levels of malondialdehyde (MDA) which is the end-product of reactions between reactive oxygen species and lipids and is therefore measure of oxidative damage.

2.3.1 Brain Homogenisation

The heads of adult *Drosophila melanogaster*, for each experimental condition, were homogenised to produce lysates. For each experimental condition, adult heads were collected at 10 and 45 days old and were placed into a labelled 1.5 ml microcentrifuge tube and stored at -80 °C.

To prepare the lysates for the lipid peroxidation assay, 16 adult heads were placed into a 1.5 ml microcentrifuge tube and 300 µl of lysis buffer (NaCl 70 mM, KCl 5mM, MgCl₂ 20 mM, CaCl₂ 1.5 mM, NaHCO₃ 10 mM, Trehalose 5 mM, Sucrose 115 mM, HEPES 5 mM, pH 7.2) was added to each tube. The sample was then homogenised using a homogeniser (Kontes, Pellet Pestle Motor) for $\cong$ five minutes until the sample became

clear and there were no visible particles. The samples were then centrifuged (MiniSpin Plus, Eppendorf) for 10 min at 12000 rpm. The supernatant was collected, ensuring that the pellet was not disturbed. The supernatant, otherwise referred to as the lysate, was then stored at -80 °C.

This same process was repeated to obtain lysates for the SOD assay (Buffer: 1x SOD Buffer).

2.3.2 Bradford Assay

To run the Bradford assay, first a standard curve was prepared with 10 µl of each standard in a clear-bottomed 96-well microplate (Nunc Surface). This was done in triplicates for each standard. A blank sample in triplicate was also included. For sample measurements, 10 µl of each lysate sample was pipetted three times, into different wells. 150 µl of the Protein assay reagent (Thermo scientific, Pierce 660 nm Protein Assay Reagent) was then added to each well. The plate was covered and was placed on a rocker (Thermomixer F1.5, Eppendorf), set to medium speed, for 1 minute. The plate was then incubated at room temperature for 5 minutes. Following incubation, the plate was run using (Clariostar) and the absorbance of the standards and samples was measured at 660 nm.

2.3.3 Superoxide Dismutase Assay

The 1x SOD buffer was prepared in deionised (DI) H₂O from the 10x SOD buffer. Then 150x xanthine oxidase (XO) was diluted to 1x XO in 1x SOD buffer. A master mixture (5 µl Xanthine solution/5 µl Chromagen solution/10 µl 10x SOD buffer/60 µl DI H₂O)(Table 5) was created and adjusted for the required number of wells. All wells were set up in triplicate and contained 90 µl in total volume (80 µl master mixture and 10 µl sample)(Table 5).

Component	Volume per well
Xanthine solution	5 µl
Chromagen solution	5 µl
10x SOD buffer	10 µl
DI H ₂ O	60 µl
Sample	10 µl
Total	90 µl

Table 5: Composition of the SOD Assay.

The contents in each well on the microplate in the SOD Assay.

Using a clear-bottomed 96-well microplate, set up a blank well was set up with 10 µl DI H₂O and 80 µl of the master mix. Standard curve wells were set up with 80 µl of the master mix per well as well as 10 µl from tubes 1-8 as shown in the table below (Table 6).

Tube Number	SOD standard (µl)	1X SOD buffer (µl)	SOD (activity/µl)
1	25	0	5
2	10 µl of tube #2	30	1.25
3	10 µl of tube #2	30	0.312
4	10 µl of tube #3	30	0.078
5	10 µl of tube #4	30	0.0195
6	10 µl of tube #5	30	0.0048
7	10 µl of tube #6	30	0.0012

Table 6: Setup of the Reference Curve Standards

The setup of each of the standards for the reference curve, to which 80 µl of master mix would be added.

The sample wells were then set up by pipetting 10 µl of each sample into their respective wells and then 80 µl of the master mix was added to each well. The

microplate was covered and incubated for one hour at 37 °C. Following incubation, the absorbance was measured at 490 nm using the plate reader (Clariostar).

In order to get results for the SOD assay, the SOD activity in units/ml needed to be calculated. This was done by calculating the average absorbance of each standard, sample, and control and then subtracting the blank value from all standards, samples, and controls values. The linearised SOD rate was then obtained by dividing the No SOD absorbance (maximal absorbance) by itself and by all other blank-subtracted standards, samples and controls absorbance readings. The SOD standard concentrations (in units/ml) were then plotted against the linearised SOD rate of all standards. The activity of the SOD in each sample was then calculated using the following equation:

$$\text{SOD Activity (units/ml)} = \frac{(\text{sample LSR} - \text{intercept})}{\text{slope}} \times 10 \times \text{DF}$$

Where:

Sample LSR = linearised SOD rate of the sample

DF = sample dilution factor

(Sigma-Aldrich Superoxide Dismutase Activity Assay Kit)

2.3.4 Lipid Peroxidation

To create the standard curve for the MDA Assay, 10 µl of the 4.17 M MDA Standard Solution was diluted in 407 µl of DI H₂O to give a 0.1 M MDA solution. 20 µl of the 0.1 M MDA Standard Solution was then further diluted with 980 µl of DI H₂O to give a 2 mM MDA Standard. 100 µl of the 2 mM MDA Standard Solution was added to 900 µl of DI H₂O to make a 0.2 mM MDA Standard Solution. Following this step, 0, 2, 4, 6, 8, 10 µl of the 0.2 mM MDA Standard Solution was pipetted into separate 1.5 ml microcentrifuge tubes. DI H₂O was then added to each tube to bring the total volume in each tube up to 200 µl which meant that 0 (blank), 0.4, 0.8, 1.2, 1.6 and 2.0 nmol standards were generated.

A thiobarbituric acid (TBA) solution was created by dissolving the preweighed bottle in 7.5 ml glacial acetic acid which was then adjusted to a total volume of 25 ml by adding DI H₂O. The solution was stored at 4 °C and was used within one week of preparation. To create samples, 200 µl of the lysate for each experimental condition was pipetted into a separate 1.5 ml microcentrifuge tube. An MDA-TBA adduct was then created by adding 600 µl of the TBA solution into each tube containing the standards and the samples. The tubes were incubated at 95 °C for 60 minutes. After incubation, the tubes were cooled to room temperature, in an ice bath, for 10 minutes. 200 µl from each tube was then pipetted into a clear-bottomed 96-well microplate for analysis, with each sample and standard being done in triplicate. The absorbance was measured at 532 nm (A_{532}) using the Clariostar.

2.4 Immunohistochemistry

2.4.1 Solution Preparation

20x phosphate buffered saline (PBS) solution was prepared by adding 25 ml 20x PBS to 475 ml DI H₂O after which the pH was adjusted to 8.0. Paraformaldehyde was diluted in 1x PBS to give a 4 % paraformaldehyde solution (PFA). The blocking and antibody dilution buffer (1x PBS/ 5 % normal serum [NGS]/ 0.3 % Triton X-100) was prepared by adding 0.5 ml normal goat serum (NGS), from the same species as the secondary antibody, and 0.5 ml 20x PBS to 9.0 ml DI H₂O. Then 30 µl of the Triton X-100 was slowly added, whilst stirring, to give the blocking buffer.

2.4.2 Brain Dissection

This method of *Drosophila melanogaster* brain dissection can produce brains with well-preserved morphology, suitable for whole mount analysis, which can be used to study the effects of genetic manipulations of neurons and brain circuitry (Tito et al., 2016). Brain dissection of *Drosophila melanogaster* was performed at either 10 or 45 days old. Each dissection was performed on a fly head that was removed from the body and

which was then immersed in cold dissecting haemolymph-like solution 3 (HL3) with the top of the head facing downwards. All dissections were performed using a dissecting microscope (Zeiss, Germany), on the highest magnification, on a silicone bottomed plate using dissection tweezers (Dumont, Switzerland).

Using a pair of tweezers in each hand, one tip of each tweezer was inserted into the small hole at the neck joint. Pressure was exerted on each pair of tweezers to close the tips, and care was taken to not penetrate too deeply and damage the brain. In one motion, each pair of tweezers was steadily pulled in opposite directions to tear open the exoskeleton of the fly head (Figure 8). The exoskeleton, head case and associated eye and trachea were then gently removed from the brain tissue. The full brain was then visible and remaining accessory tissues were then carefully removed. The intact brain was then carefully pipetted up, using a P20 pipette with the tip of the pipette tip cut off, and was placed into 30 μ l of 4 % paraformaldehyde solution (PFA).

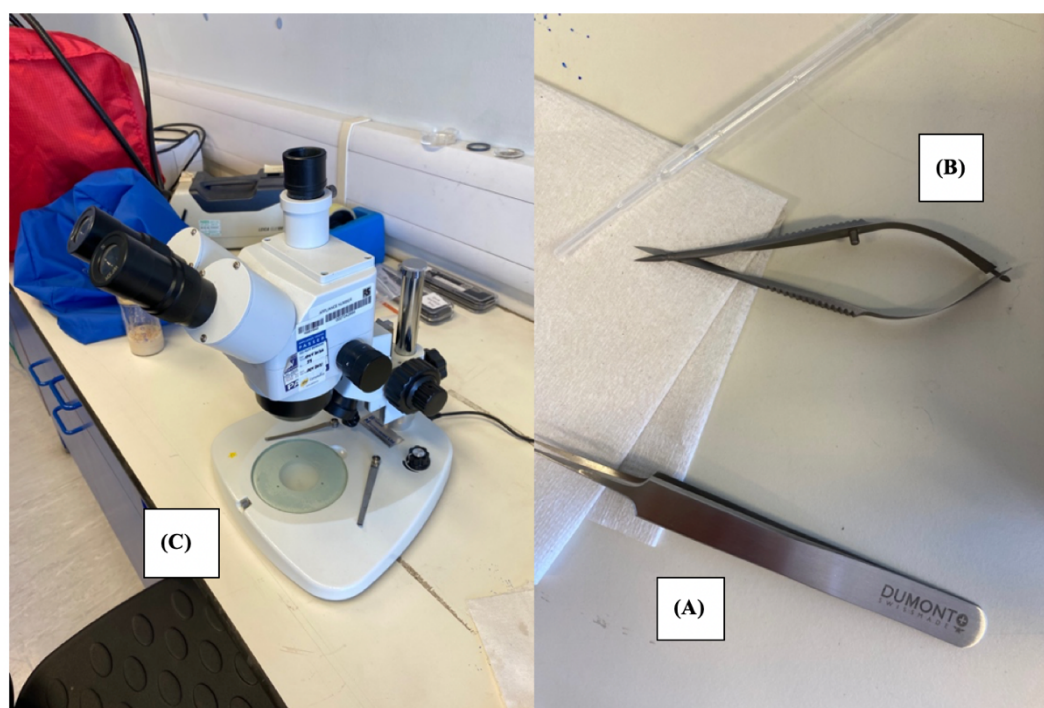


Figure 8: *Drosophila melanogaster* Brain Dissection Equipment.

Drosophila melanogaster brain dissection required use of sharp dissection tweezers (A), sharp dissection scissors (B), and a dissecting microscope (C).

2.4.3 Specimen Preparation

Dissected *Drosophila* brains were individually immersed, post-dissection, in 30 µl of 4 % paraformaldehyde solution (PFA) in a 1.5 ml microcentrifuge tube. This was done inside a fume hood and the specimens were then incubated, in the fume hood, at room temperature for 20 minutes to allow the tissue to fix. Following this, the PFA was removed, using a pipette, and 30 µl 1x PBS was added. The tubes were placed on a rocker for 10 minutes to wash the brain in each tube. The PBS was removed, using a pipette, and the washing step was repeated two more times, to give 3 washes in total, using fresh 30 µl 1x PBS each time. Following the last wash, the 1x PBS was removed from the tube, using a pipette, and the specimen was prepared for blocking.

2.4.4 Immunostaining and Slide Preparation

The cleaved Caspase-3 antibody is a popular tool in apoptosis research in *Drosophila melanogaster* (Fan and Bergmann, 2010). The cleaved caspase-3 antibody can be used in the *Drosophila melanogaster* brain, using immunohistochemical techniques, to detect cleaved Caspase-3-like proteins. This method was used in this project to show any diet-, age- or genotype-related changes in the amount of neuronal apoptosis in the *Drosophila* brain (Fan and Bergmann, 2010).

The specimen in each microcentrifuge tube was blocked in 30 µl blocking buffer (1x PBS/ 5 % normal goat serum [NGS]/ 0.3 % Triton X-100) for 60 minutes. During blocking, the primary antibody (Cell Signalling Technology, Caspase-3 rabbit antibody) was prepared by diluting 1 µl of the antibody in 399 µl of the blocking buffer (1:400). After 60 minutes, the blocking solution was removed from the tube, using a pipette, and 30 µl of the diluted primary antibody was added to each tube. The contents of each vial were then incubated overnight at 4 °C.

Following incubation, the primary antibody was removed, using a pipette, and 30 µl 1x PBS was added to each tube. The tubes were then placed on a rocker for 10 minutes to wash the brain in each tube. The PBS was removed, using a pipette, and the washing

step was repeated two more times, to give 3 washes in total, using fresh 30 µl 1x PBS each time.

During the last wash, the secondary antibody (Invitrogen, Alexa Fluor 647 goat anti-rabbit antibody IgG) was prepared by diluting 1 µl of the antibody in 399 µl of the blocking buffer (1:400). 30 µl of the diluted secondary antibody was added to each of the tubes, which were then incubated for 1-2 hours, at room temperature, in the dark. Following incubation, the secondary antibody was removed, using a pipette, and 3 more washes in 30 µl 1x PBS were performed, on a rocker, using fresh 30 µl 1x PBS each time.

Each brain was pipetted up, using a P200 pipette, and was placed on a clean microscope slide. At this step, it was found that cutting off the tip of the pipette tip, using sharp scissors, prevented the brain from sticking to the pipette tip. The brain on the slide was briefly looked at under the dissecting microscope to affirm that the brain was oriented correctly on the slide. Then, using a P10 pipette, excess PBS was removed from around the brain on the slide. A drop of Prolong Gold Antifade Reagent with DAPI (Sigma, Fluoroshield with DAPI) was released onto the brain. A coverslip was then carefully placed down over the brain at an angle, to avoid air bubbles, and the edges of the coverslip was sealed using clear nail varnish. The slides were horizontally stored at 4 °C in a dark slide box, protected from light, overnight.

2.5 Confocal Imaging

The slides were imaged using Confocal microscope (Zeiss LSM 880). The number of neuronal cells undergoing apoptosis was identified by the extent of immunofluorescence in cells labelled with the caspase-3 antibody. Z-stacked images of the full brain at 20x magnification were initially captured. Additionally, the mid-brain region was focused on and Z-stacked images at 40x magnification were captured at 365 nm (DAPI) and 633 nm (Alexa Fluor 647) excitation. All images were quantified following constructions of maximal intensity projections in ImageJ (v 1.53) to generate a maximal intensity of images (DAPI and cleaved Caspase-3) to be quantified.

2.6 Longevity Assay

Following the same experimental set up, discussed above, a minimum of 30 flies were separated at 5 days old into their respective experimental groups to give 24 different experimental conditions according to age, sex, diet, and genotype (Table 3). This set up was repeated to give a minimum of 4 vials, of 30 flies in each vial, per experimental condition. This meant that for each experimental condition in the longevity assay, there were at least 120 flies per condition. The food in each vial was changed weekly and fly vials were kept at 18 °C. The number of flies in each vial was counted every day or every second day and was recorded until all flies were dead. This data was then used to generate life span curves for comparison.

2.7 Climbing Assay

A climbing assay protocol was created to observe mobility and motor function. The testing chamber for the assay was composed of a vial, with markings at five centimetres and eight centimetres, and a sponge stopper (Figure 9). The vials were labelled according to the experimental group of flies in the vial. Polystyrene shields were used to block off natural light from the sides of the tubes as this can alter climbing behaviour (Figure 9).

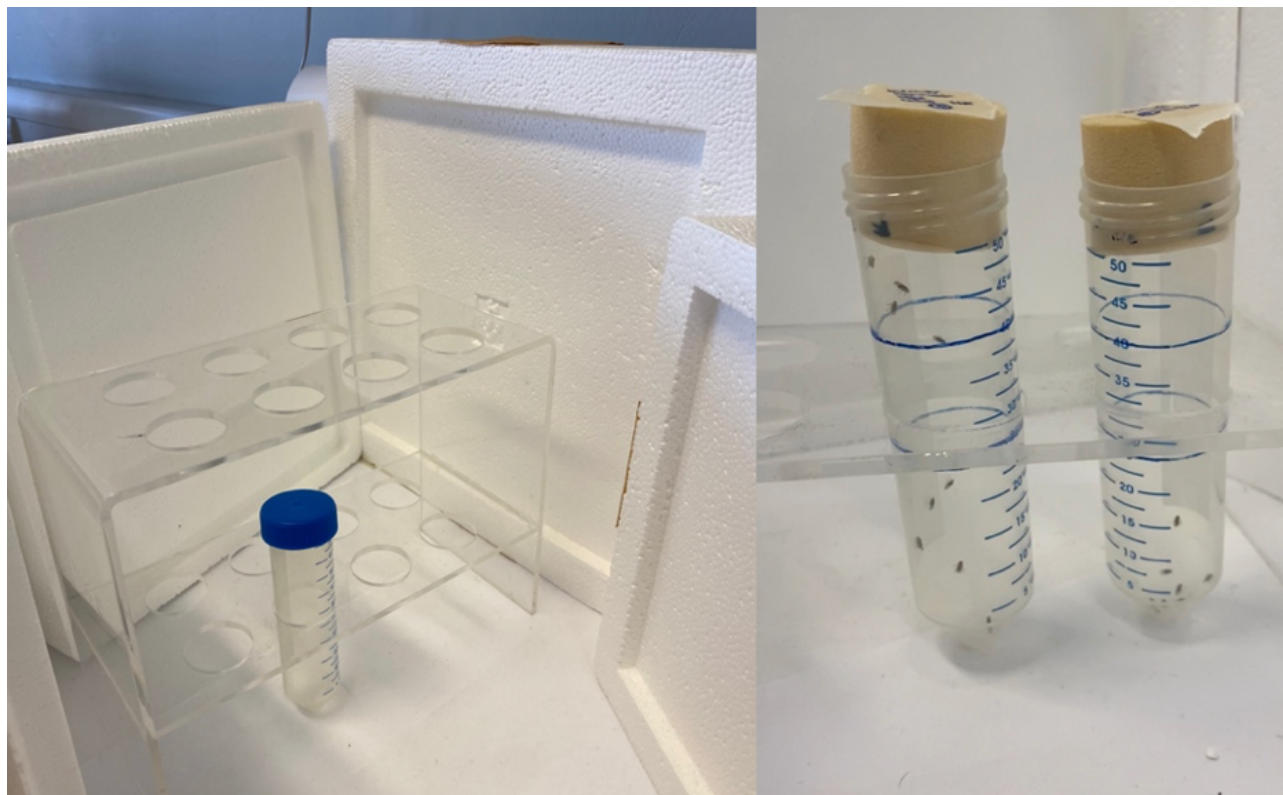


Figure 9: Set-up of Climbing Assay.

Set-up of the climbing assay with polystyrene shields to block off natural light from the sides of the falcon tubes.

Male and female vials were labelled and kept separately. Additionally, after each climbing assay for an experimental group was completed, the vials were washed with ethanol and then rinsed with distilled water to prevent the chance of odour affecting climbing behaviour.

Ten flies were transferred to the testing chamber vials, using carbon dioxide anaesthesia, and were left to rest for 30 minutes to minimise the effect of anaesthesia on climbing ability. Following this resting period, the flies were tapped to the bottom of the vial and the number of flies above the eight centimetre mark after eight seconds was recorded. The flies were allowed to recover for an additional minute and then the assay was repeated. This was done three times to generate three different recordings.

Additionally, this set up was repeated three times for each experimental condition. This allowed for an average (average number of flies above the eight centimetre mark after eight seconds) to be created for each of the three vials for each experimental condition, which then allowed for a total average for each condition to be calculated from the average of each of the three vials. The climbing assay data for each experimental group was used to generate a graph which allowed for comparison of motor function between experimental conditions.

2.8 Statistical Analysis

Statistical analysis of the data from the climbing ability, longevity, IHC quantifications, SOD assay and MDA assay were conducted using GraphPad Prism v10 (GraphPad Software Inc., San Diego, CA, USA). A two-way ANOVA test was used when applicable with a posterior test (with Tukey's multiple comparison). After confirming normal data distributions (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test), parametric statistics was applied. Cumulative survival curves are presented and compared using the log-rank (Mantel-Cox) test. Data are expressed as mean $\pm$ SEM where n is the number of flies/brains as indicated. Significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3 Results and Discussion:

3.1 Superoxide Dismutase Assay

3.1.1 Results

To investigate the effect of diet, age, and genotype on oxidative stress in AD, we assessed the activity of SOD in all experimental groups. We compared age-, diet- and genotype-dependent changes in three different genotypes, A β arc, hApoE4 and W₁₁₁₈. It is important to note that for this assay, only data from the male experimental groups was used as insufficient data for the female experimental groups was acquired. Figure

10 shows SOD activity, in units/ μ g protein, for each experimental group which is a measure of the oxidative stress response in the *Drosophila* brain. Two-way ANOVA comparisons revealed that SOD activity was significantly altered in an age- and diet-dependent manner (diet: $F(5, 24) = 12783$, $p < 0.0001$ and age: $F(1, 24) = 1108$, $p < 0.0001$, Figure 10). Comparisons between genotypes show increased SOD activity in wildtype flies, compared to AD genotypes, indicating genotype-dependent effects on SOD activity (Figure 10). Additionally, Figure 10 shows a slight increase in SOD activity in the NF experimental groups compared to the HF experimental groups, apart from NF and HF 10d wildtype flies and NF and HF 45d A β arc flies. However, these diet-related differences were minimal.

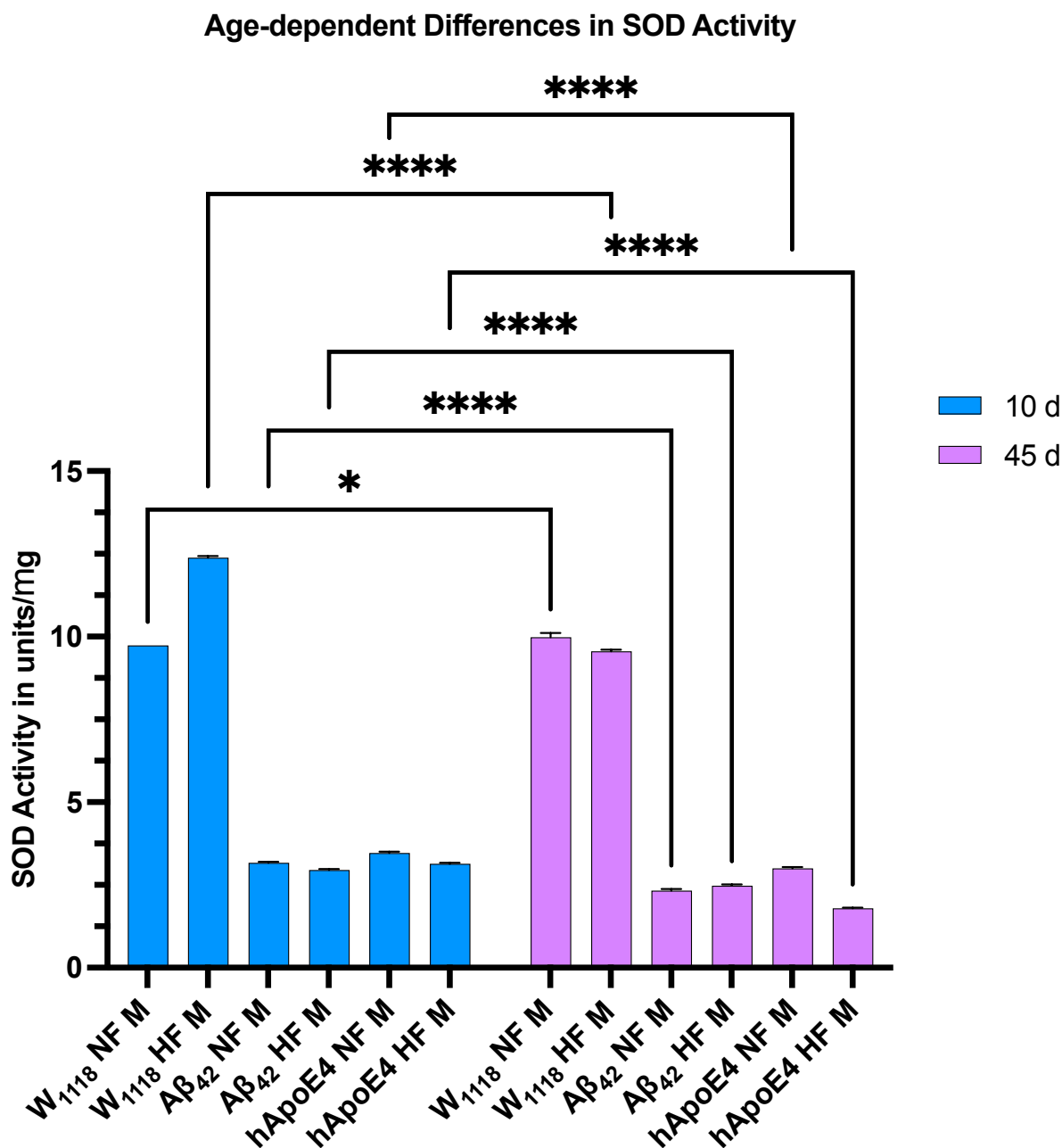


Figure 10: SOD Activity is Reduced in AD Models.

SOD activity was assessed in male (M) fly heads at 10 and 45 days (d) of age. Both Aβ₄₂ and hApoE4 expressing flies displayed reduced SOD activity at 45 days ($p < 0.0001$, Two-way ANOVA, $n=16$ heads per condition). Significance is shown as * $p < 0.05$ and **** $p < 0.0001$.

Two-way ANOVA comparisons for each genotype were conducted separately to assess changes in age- and diet-dependent effects more directly within the different genotypes.

These comparisons showed statistically significant age- and diet-related differences for the W_{1118} and hApoE4 genotypes. The two-way ANOVA comparison for the wildtype experimental groups, showed statistically significant age and diet related differences (diet: $F(1,8) = 504.6$, $p < 0.0001$ and age: $F(1, 8) = 122.4$, $p < 0.0001$, Figure 11A). Similarly, the comparison for hApoE4 showed significant changes related to both age and diet (diet: $F(1,8) = 598.0$, $p < 0.0001$ and age: $F(1, 8) = 827.1$, $p < 0.0001$, Figure 11C). In A β arc flies, the diet-dependent differences were seen as less significant and showed less changes between those fed a NF diet and those fed a HF diet (Figure 11B).

Interestingly, in the wildtype flies SOD activity increased with age in NF but decreased with age under HF conditions ($p < 0.0001$, Figure 11A). However, a HF diet alone was seen to enhance SOD levels in male wildtype flies at 10d ($p < 0.0001$, Figure 11A). At 45d, SOD levels were higher in the wildtype flies fed NF than those fed a HF diet ($p < 0.01$, Figure 11A).

In hApoE4 flies, SOD activity decreased with age and were significantly lower in the HF group compared to the NF group ($p < 0.0001$, Figure 11C). SOD levels in the A β arc flies were elevated at 10d, regardless of diet, compared to 45d ($p < 0.0001$, Figure 11B). At 10d SOD levels were more elevated in the A β arc flies fed a NF diet than those fed a HF diet ($p < 0.01$, Figure 11B).

Age- and Diet-dependent Differences in SOD Activity by Genotype

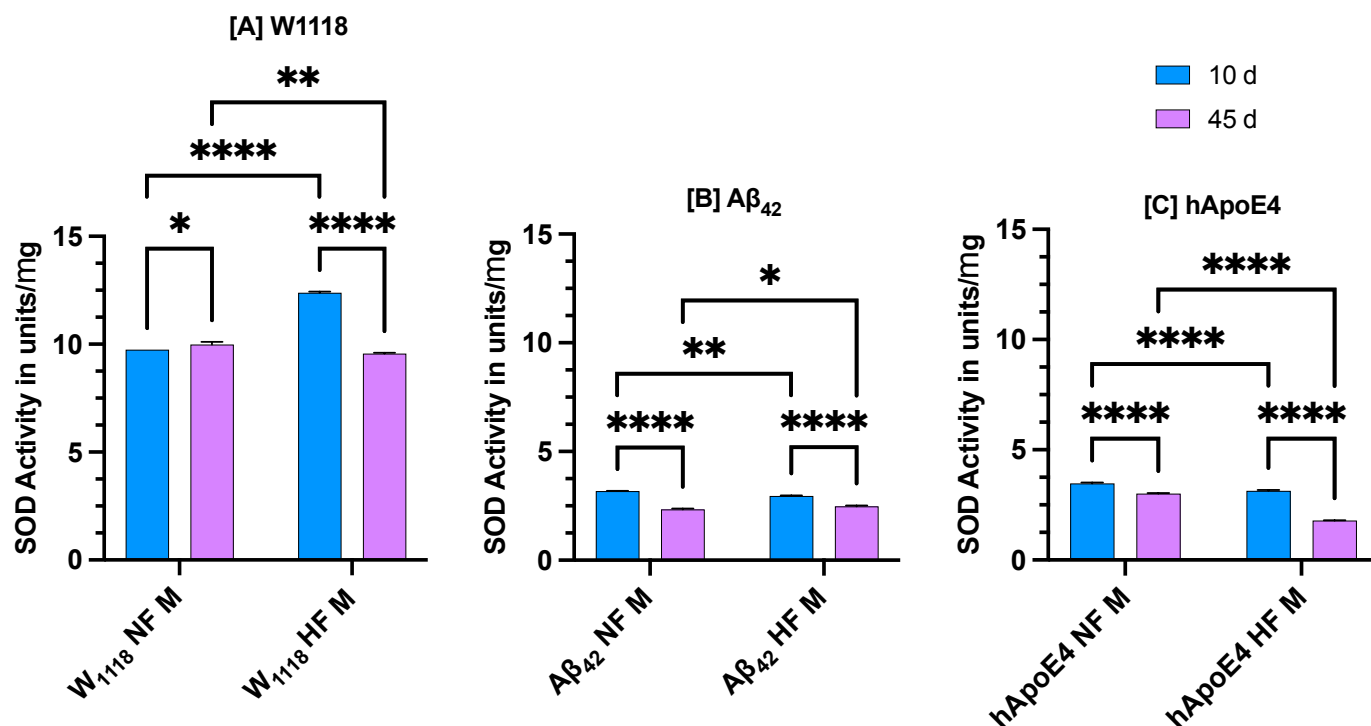


Figure 11: SOD Activity for Each Genotype.

SOD activity was assessed in male (M) fly heads at 10 and 45 days (d) of age. **[A]** SOD activity in 10d and 45d old W₁₁₁₈ flies that were fed either a HF or NF diet. **[B]** SOD activity in 10d and 45d old A β arc flies that were fed either a HF or NF diet. **[C]** SOD activity in 10d and 45d old hApoE4 flies that were fed either a HF or NF diet. Significant age- and diet-related differences in hApoE4 and W₁₁₁₈ flies ($p < 0.0001$, Two-way ANOVA, $n = 16$ heads per condition) were observed. Significance is shown as * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

3.1.2 Discussion

This data is an indirect measure of oxidative stress in the *Drosophila* brain by measuring the activity of SOD. The data showed that there were significant age- and diet-related changes between all three genotypes with aged flies exhibiting decreased SOD activity (Figure 10). Comparisons between the three different genotypes, A β arc, hApoE4 and W₁₁₁₈, show higher SOD activity in wildtype flies, compared to both AD genotypes (Figure 10). This was expected as the W₁₁₁₈ flies served as the control group and demonstrated a 'normal' oxidative stress response during ageing compared to the two AD genotypes. The data showed that genotype had the most significant effect on SOD activity. The effect of diet on SOD activity was minimal, and was mainly observed

in W_{1118} and hApoE4. This was expected as Trindade de Paula et al., (2016) showed no difference in SOD activity in a 10% HF diet.

The age-related changes to all experimental conditions were as anticipated given that SOD activity generally decreases with age as the oxidative stress response is affected. For most experimental conditions, this decrease in SOD activity with age was observed apart from W_{1118} NF male flies where the opposite was seen.

The assessment of data by genotype allowed for more direct analysis of SOD activity in A β arc, hApoE4 and W_{1118} experimental conditions given that the most notable differences were genotype related. In W_{1118} , the control group, the data suggested an elevated oxidative stress response, higher SOD activity, at 10d in a HF diet compared to that in a NF diet however the opposite was noted in 45d flies (Figure 11A). The 10d W_{1118} flies fed a HF diet showed a greater oxidative stress response than those fed a NF diet, which was expected, however this was not seen in the HF and NF 45d W_{1118} flies (Figure 11A). The results in the W_{1118} flies do not thoroughly indicate whether age or diet play a significant role in impairing the oxidative stress response.

In the A β arc conditions, SOD activity was affected by natural ageing as the 10d flies showed a greater oxidative stress response than the 45d flies (Figure 11B). Diet-dependent differences were more noticeable in the 10d old flies, most likely because their response to oxidative stress is less affected by ageing, and there was almost no diet-dependent differences in 45d flies (Figure 11B). Figure 11B does not indicate that diet plays a significant role in affecting SOD activity in A β arc flies.

The hApoE4 data, showed lower levels of SOD in 45d flies which follows our understanding that SOD activity decreases with age (Figure 11). Additionally, there was statistically significant decreased SOD activity in 10d and 45d HF groups when compared to their respective NF control groups (Figure 11C, $p < 0.0001$). The decreased SOD activity response in the hApoE4 flies suggests age and diet do not increase ROS formation in the brain, since SOD activity and ROS have a directly

proportional relationship in the brain of AD flies as described in Ali et al., 2019 (Figure 11C, $p < 0.0001$). However, these results could suggest that SOD response is impaired with age (Trindade de Paula et al., 2016).

The most evident differences in the SOD assay results were genotype-related (Figure 10). The data showed significantly higher SOD activity in W_{1118} flies compared to the two AD genotypes, hApoE4 and A β arc (Figure 10 and 11A-C). This was anticipated as these are not the diseased flies and would therefore most likely not have an affected oxidative stress response. Both A β arc and hApoE4 flies showed reduced SOD activity (Figure 10 and Figure 11B and 11C).

In order to fully investigate the interplay of oxidative stress and SOD response, the levels of ROS should be tested. The most significant difference in the results were age- and genotype-related. There were minimal diet-related differences in the results from which to draw definitive conclusions and therefore the effects of a HF diet on SOD activity needs to be further investigated. It was expected that a HF diet would either reduce ROS resulting in decreased SOD activity or hinder the response to oxidative stress such that SOD activity is affected (Ali et al., 2019). Additionally, the data does not conclusively suggest that decreases in SOD activity, in flies fed a HF diet, is a direct result of the diet since it is not known whether this decrease is due to an affected oxidative stress response, the result of decreased oxidative stress, or a complicated interplay between age, genotype and diet (Ali et al., 2019; Sundararajan et al., 2021; Alamro et al., 2020). Prior research did suggest that coconut oil may have anti-oxidant and anti-inflammatory properties (Mirzaei et al., 2018). This provides additional evidence that a HF diet needs to be investigated further as a HF diet could be interpreted as having neuroprotective effects, which would affect SOD activity. However, this research from Mirzaei et al. (2018) was conducted in rats and therefore may not be transferable to *Drosophila melanogaster*.

3.2 Lipid Peroxidation Assay

3.2.1 Results

Levels of lipid peroxidation, in the *Drosophila* brain, in all experimental groups were investigated through comparison of age- and diet-dependent changes in three different genotypes, A β arc, hApoE4 and W₁₁₁₈. Figure 12 shows quantity of MDA, in nM/ μ g protein, for each experimental group which is an end-product of lipid peroxidation and therefore a measure of oxidative damage. Two-way ANOVA comparisons revealed that the amount of MDA was altered, with statistical significance, in an age- and diet-dependent manner (diet: $F(5, 24) = 978.6$, $p < 0.0001$ and age: $F(1, 24) = 47.24$, $p < 0.0001$, Figure 12).

For wildtype flies, MDA decreased with age regardless of diet, apart from the female NF experimental group ($p < 0.0001$). In A β arc flies, there were no obvious trends observed other than MDA decreasing with age ($p < 0.0001$, Figure 12B). This trend was seen in all conditions, regardless of diet, apart from A β arc HF female group where MDA increased with age ($p < 0.0001$, Figure 12B).

The hApoE4 flies displayed an opposite trend to the wildtype flies with MDA increasing with age regardless of diet ($p < 0.001$, Figure 12C). Similarly, the female NF experimental group was opposite to this general trend with MDA decreasing with age ($p < 0.01$). In 10d conditions, MDA was increased in flies fed a NF diet compared to those fed a HF diet (Female: $p < 0.0001$, Male: $p < 0.01$, Figure 12C). The opposite was exhibited in the amount of MDA in 45d conditions where MDA was decreased in flies fed a NF diet compared to those fed a HF diet (Female: n/s, Male: $p < 0.01$, Figure 12C).

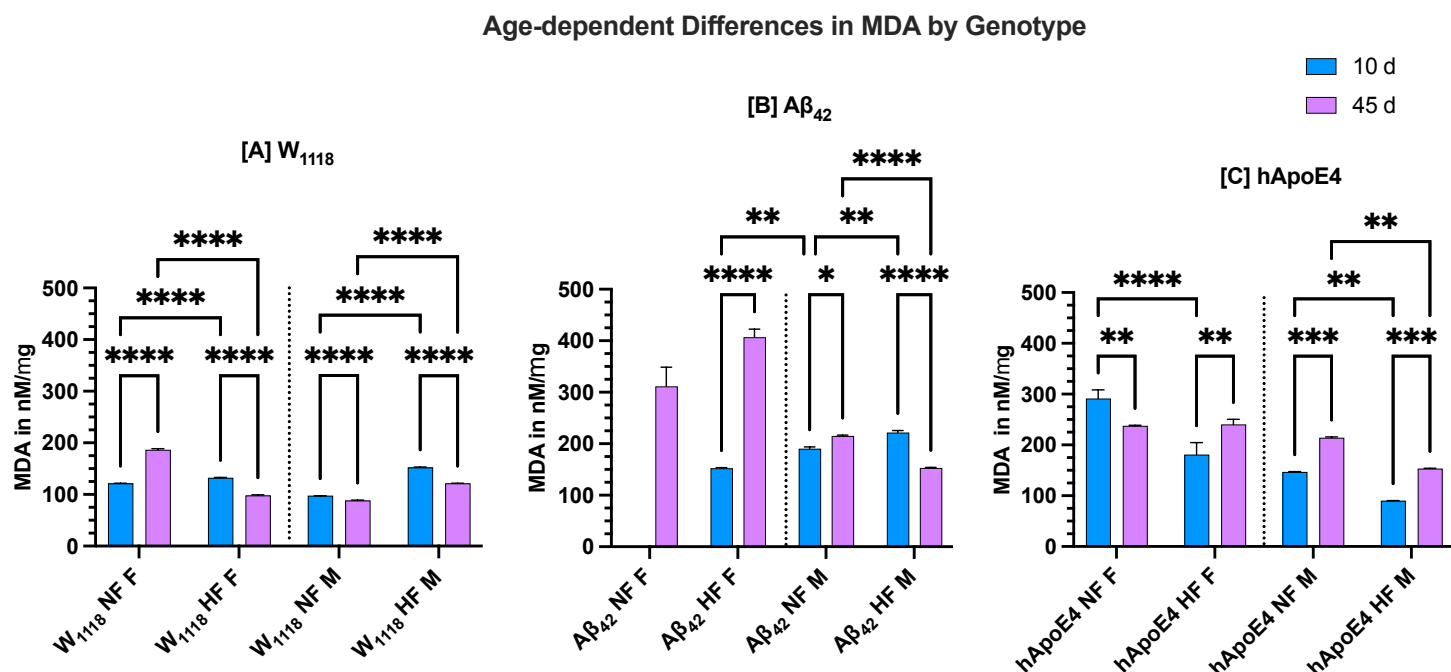


Figure 12: Quantification of MDA Levels in Each Condition by Genotype.

MDA levels in three different *Drosophila* genotypes were assessed. [A] MDA levels in 10d and 45d old male and female W_{1118} flies that were fed either a HF or NF diet. [B] MDA levels in 10d and 45d old male and female $A\beta_{42}$ flies that were fed either a HF or NF diet. [C] MDA levels in 10d and 45d old male and female hApoE4 flies that were fed either a HF or NF diet. Age- and diet-dependent changes were exhibited (diet: $F(5, 24) = 978.6$, $p < 0.0001$ and age: $F(1, 24) = 47.24$, $p < 0.0001$, Two-way ANOVA, $n = 16$ heads per condition). Significance is shown as ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3.2.2 Discussion

For wildtype flies, MDA was observed to decrease with age regardless of diet or sex (Figure 12A). The only exception to this trend was the female NF experimental group where the amount of MDA was seen to increase with age (Figure 12A). It was expected that MDA, which is a measure of lipid peroxidation, would increase with age in wildtype flies as oxidative stress increases (Kizhakke et al., 2019; Ali et al., 2019). Therefore, the decrease in MDA with age was an unexpected result. Moreover, the results showed that MDA increased in wildtype 10d HF and 45d male HF experimental groups compared to their respective NF conditions (Figure 12A). This result was hypothesised as it was expected that a HF diet would increase oxidative stress and thereby exhibiting a higher amount of MDA (Ali et al., 2019; Kizhakke et al., 2019; Trindade de Paula et al., 2016).

The opposite occurred in the female 45d experimental groups where MDA decreased in the HF conditions. Interestingly, in W_{1118} conditions, the exceptions to noticeable trends in the results were female experimental groups (Figure 12A).

The A β arc results for the MDA assay did not offer as obvious trends, apart from the general observation that MDA increased with age (Figure 12B). This trend was exhibited in all A β arc conditions, regardless of diet, apart from the A β arc HF male group where MDA decreased with age (Figure 12B).

The hApoE4 flies displayed an opposite trend to the wildtype flies. In the hApoE flies, MDA levels increased with age regardless of diet. Similarly, in contrast to the W_{1118} MDA results, the female NF experimental group was opposite to this trend with MDA decreasing with age (Figure 12C). In 10d conditions, it was observed that MDA increased in flies fed a NF diet compared to those fed a HF diet while in 45d conditions the amount of MDA decreased in flies fed a NF diet compared to those fed a HF diet (Figure 12C). It was expected that LP would increase with age and that this would be exacerbated by a HF diet (Cormier et al., 2023; Thapa and Carroll, 2017; Trindade de Paula et al., 2016). This trend in the hApoE4 flies was opposite to that observed in the W_{1118} flies, even down to their respective opposing results in the female NF conditions. The hApoE4 MDA assay results also showed the female experimental groups as being exceptions to trends that were observed. This comparison of the results, between W_{1118} and hApoE4, was interesting to consider. In both hApoE4 and W_{1118} NF female groups, there were noticeable differences in MDA levels (Figure 11A & 11C). One possible explanation for this discrepancy could be that female flies are responsible for the production of eggs and therefore could have different fatty acid profiles, which is relevant when considering the effects of a HF diet (Cormier et al., 2023; Baenas and Wagner, 2022). This sex-related difference in MDA levels opens up an interesting question how gender may influence cellular signalling and are important to consider where diet is involved, as sex differences in response to nutritional variations have been widely reported in *Drosophila* (Baenas and Wagner, 2022).

3.3 Immunohistochemistry and Confocal Imaging

3.3.1 Results

Immunostaining of the *Drosophila* brain was performed to detect cleaved Caspase-3 in the CNS so that age- and diet-dependent factors in three different genotypes, A β arc, hApoE4 and W₁₁₁₈, could be investigated. Caspase-3 is a known key mediator of neuronal apoptosis. We quantified the immunofluorescence of cleaved Caspase-3 labelling over the entire mid-brain, as a measure of apoptosis in the mid-brain. The mid-brain was the chosen focus for this experiment meaning that the optic lobes were not assessed and therefore differences in between specific brain regions was not investigated. Additionally, due to time constraints, we did not separately characterise data from male and female flies.

Figure 13 shows a representative set of images to illustrate overviews of brain imaging and was not used for quantification purposes. These images show the full *Drosophila* brain for genotypes W₁₁₁₈ and A β arc and display DAPI and cleaved Caspase-3 immunostaining in separate images and then DAPI and cleaved Caspase-3 immunostaining overlaid in one image. Figure 14 shows images of the mid-brain of A β arc, hApoE4 and W₁₁₁₈ at two different ages, 10d and 45d old, immunostained with cleaved Caspase-3. These images serve to show the difference in apoptosis at different ages and in different genotypes. Figure 15 displays the mid-brain of A β arc, hApoE4 and W₁₁₁₈ at 45d old, immunostained with cleaved Caspase-3 (Figure 15A-C) and DAPI (Figure 15D-F). The images were generated by Z-projections of maximal intensities using ImageJ.

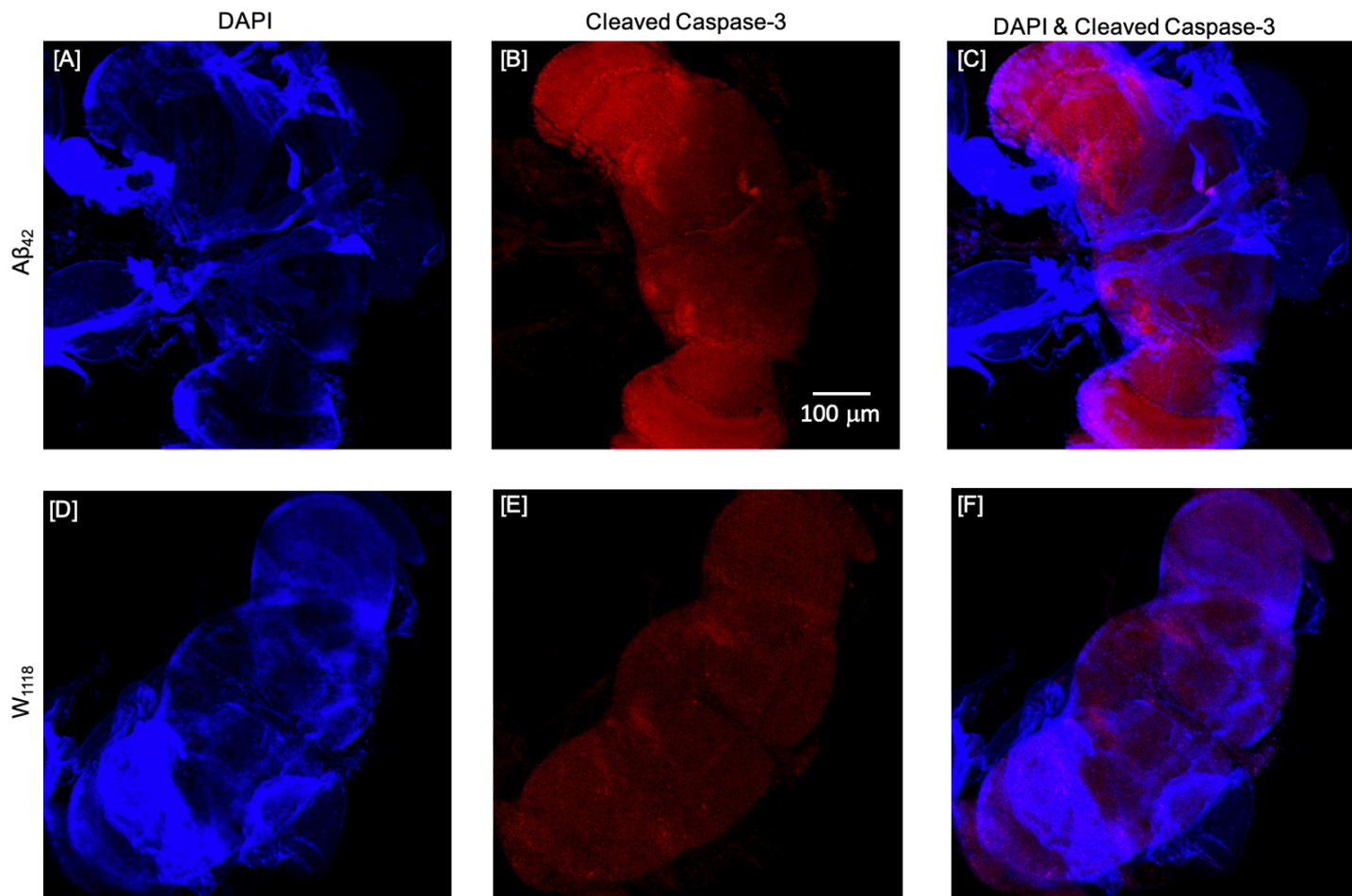


Figure 13: Confocal Images of the *Drosophila melanogaster* Brain at 20x Magnification.

The *Drosophila melanogaster* brain were immunostained using DAPI (blue) and a cleaved Caspase-3 antibody (red). Z-stack images of the full brain were captured, using the Confocal microscope (Zeiss LSM 880), at 20x magnification at 365 nm (DAPI) and 633 nm excitation. Maximal intensity projects are displayed. top A β arc, bottom W₁₁₁₈ at 10 days. This is a representative set of images to illustrate overviews of brain imaging and was not used for quantification purposes. **[A]** 10d old A β arc fly brain immunostained using DAPI at 20x magnification. **[B]** 10d old A β arc fly brain immunostained using cleaved Caspase-3 at 20x magnification. **[C]** 10d old A β arc fly brain immunostained using DAPI and cleaved Caspase-3 at 20x magnification. **[D]** 10d old W₁₁₁₈ fly brain immunostained using DAPI at 20x magnification. **[E]** 10d old W₁₁₁₈ fly brain immunostained using cleaved Caspase-3 at 20x magnification. **[F]** 10d old W₁₁₁₈ fly brain immunostained using DAPI and cleaved Caspase-3 at 20x magnification.

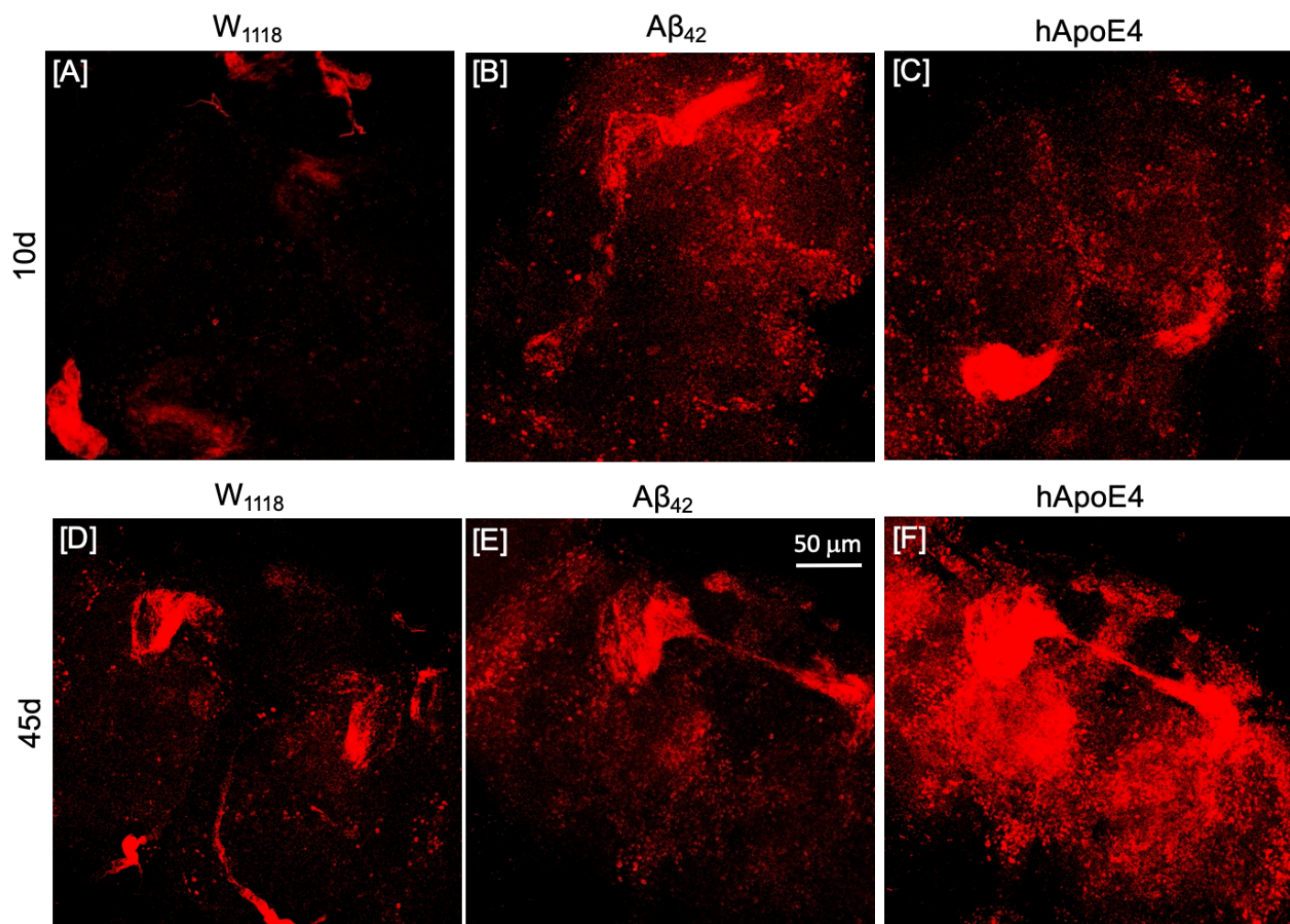


Figure 14: Caspase-3 Activity in Drosophila Brain by Genotype and Age.

Cleaved Caspase-3 was stained in the *Drosophila melanogaster* brain, in three different genotypes, at 10 and 45 days (d) of age that were fed a NF diet. Images were taken using the Confocal microscope (Zeiss LSM 880). The mid-brain region was focused on and Z-stacked images at 40x magnification were captured at 633 nm excitation. Maximal intensity projects are displayed. **[A]** 10d old W_{1118} fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[B]** 10d old $A\beta_{42}$ fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[C]** 10d old hApoE4 fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[D]** 45d old W_{1118} fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[E]** 45d old $A\beta_{42}$ fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[F]** 45d old hApoE4 fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification.

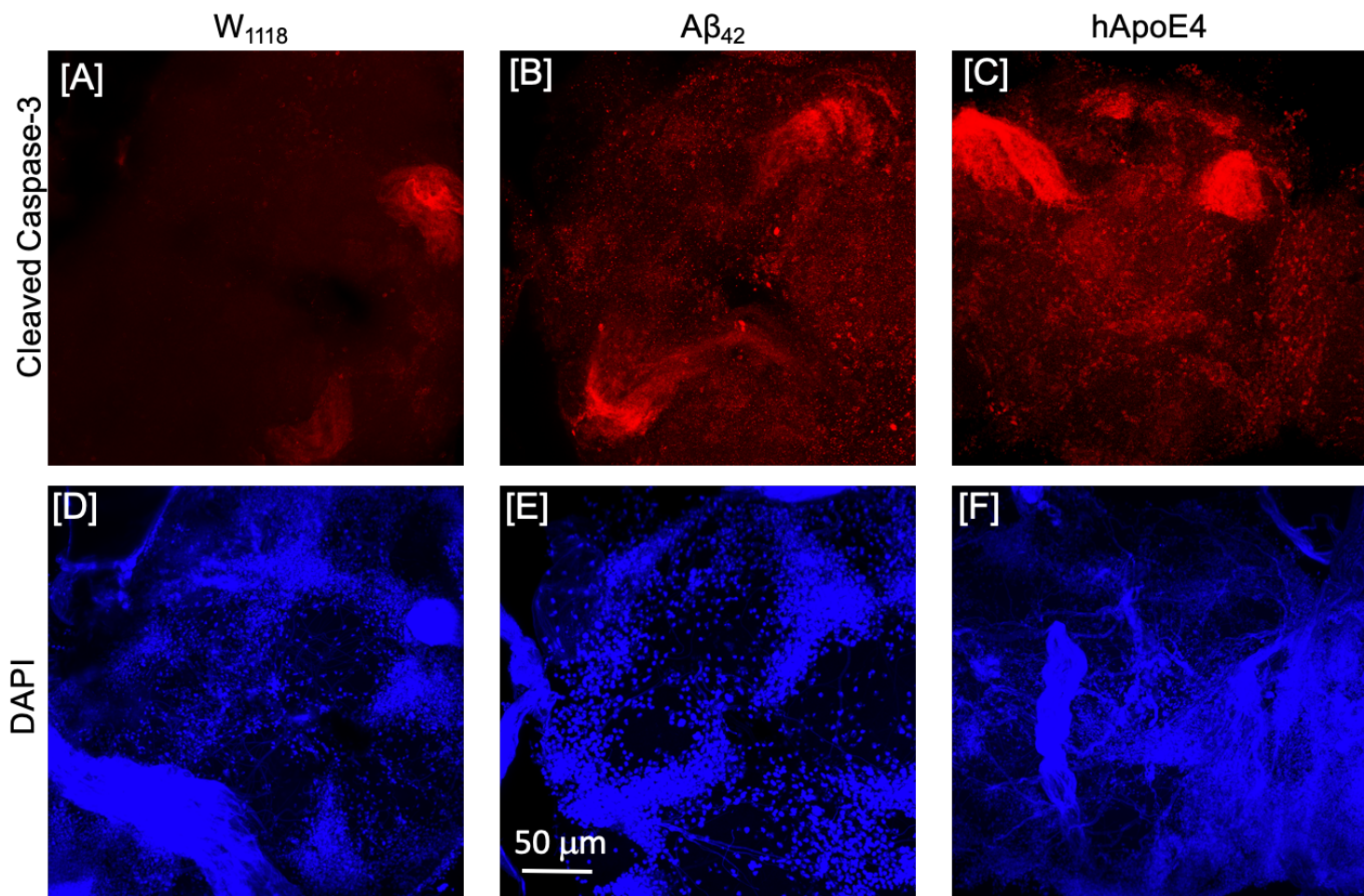


Figure 15: Confocal Images of the *Drosophila melanogaster* Brain by Genotype for 45d NF.

Immunohistochemistry of the *Drosophila melanogaster* brain, for three different genotypes, was performed at 45 days (d) of age. The tissue was stained using DAPI (blue) and a cleaved Caspase-3 antibody (red). Images were taken using the Confocal microscope (Zeiss LSM 880). The mid-brain region was focused on and Z-stacked images at 40x magnification were captured at 365nm (DAPI) and 633nm excitation. Maximal intensity projects are displayed. **[A]** 45d old W_{1118} fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[B]** 45d old $A\beta_{42}$ fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[C]** 45d old hApoE4 fly mid-brain immunostained using cleaved Caspase-3 at 40x magnification. **[D]** 45d old W_{1118} fly mid-brain immunostained using DAPI at 40x magnification. **[E]** 45d old $A\beta_{42}$ fly mid-brain immunostained using DAPI at 40x magnification. **[F]** 45d old hApoE4 fly mid-brain immunostained using DAPI at 40x magnification.

A two-way ANOVA comparison revealed that cleaved Caspase-3 activity levels were significantly altered in a diet-dependent manner (diet: $F(5, 34) = 5,288$, $p < 0.0011$, Figure 16). We found a significant increase in Caspase-3 immunofluorescence in wildtype at 10d and 45d flies that were fed a HF diet ($p < 0.001$, Figure 16).

Comparisons between genotypes displayed increased caspase-3 in A β arc 10d NF flies compared to W₁₁₁₈ 10d NF flies ($p < 0.05$) which was not affected further by age or diet. Most strikingly, 45d hApoE4 flies that were fed a HF diet exhibited increased Caspase-3 activity compared to 45d A β arc HF diet and hApoE4 flies fed a NF diet ($p < 0.01$, Figure 16).

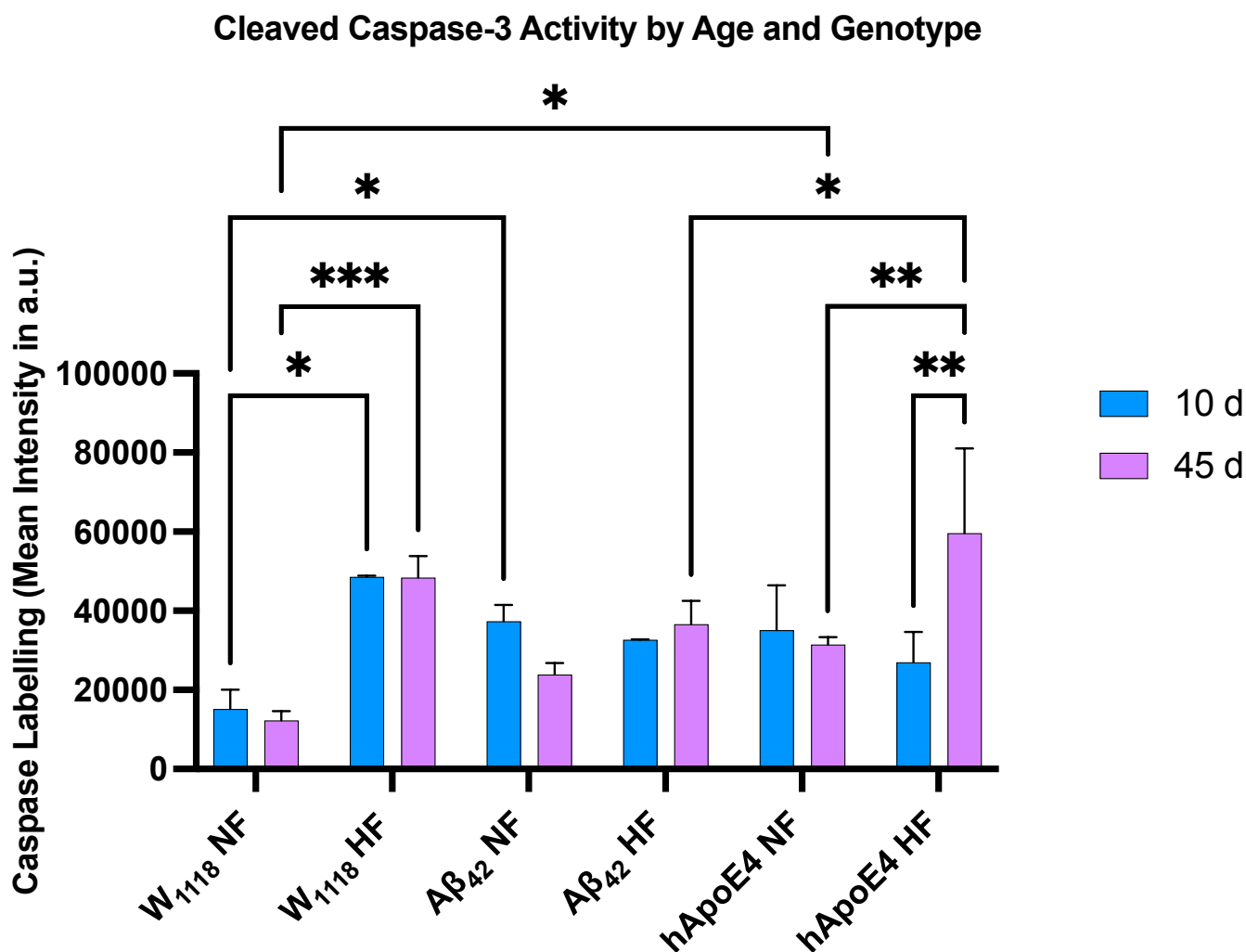


Figure 16: Caspase-3 Labelling in *Drosophila melanogaster* Brain by Genotype and Age.

The *Drosophila melanogaster* brain, of three different genotypes, were immunostained to detect cleaved Caspase-3 in neuronal cells. Immunohistochemistry was performed at 10 and 45 days (d) of age. Caspase-3 activity was altered significantly in a diet-dependent manner (diet: $F(5, 34) = 5,288$, $p < 0.0011$, Two-way ANOVA). Significance is shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The only significant age-dependent difference that was observed was an increase in Caspase-3 in 45d hApoE4 flies compared to 10d hApoE4 flies, both fed a HF diet ($p < 0.01$, Figure 16) which may not be surprising as both AD strains show already markedly elevated levels at 10d.

3.3.2 Discussion

Immunohistochemistry of the *Drosophila* brain was conducted to detect Caspase-3 in the CNS so that age- and diet-dependent factors in A β arc, hApoE4 and W₁₁₁₈ could be investigated. Caspase-3 is a known key mediator of neuronal apoptosis, a well characterised mechanism for neuronal death in neurodegenerative disorders (Khan et al., 2015; Fan and Bergmann, 2010). It must be noted that flies were not further grouped according to sex and both male and female brains were used for all experimental conditions, given the complexity of the dissection and the immunohistochemistry protocol. This was decided given no sex-dependent differences in brain morphology or immunoreactivity were observed in preliminary images of brains from female and male 10-day old wildtype flies.

The most noticeable difference in results appeared to be related to diet which was more pronounced in the 45d flies than in the 10d flies, suggesting an interplay between diet and age or a chronic rather than acute impact of diet (Mhatre et al., 2014; Figure 16). Evidence of this was the significant increase in Caspase-3 immunofluorescence in the control wildtype 45d flies that were fed a HF diet compared to wildtype 45d flies that were fed a NF diet. There was also a remarkable difference between W₁₁₁₈ 10d flies fed a HF diet compared to NF diet (Figure 16). These flies served as the control group and displayed significant diet-related changes, evident even from early on in age, which suggests a HF diet promotes apoptosis in the *Drosophila* brain.

Furthermore, 45d hApoE4 flies that were fed a HF diet exhibited a dramatic increase in Caspase-3 activity compared to 45d hApoE4 flies fed a NF diet, which indicated more apoptotic activity in HF flies (Fan and Bergmann, 2010; Figure 16). This is important as

hApoE4 are representative of sporadic AD where genetic and environmental factors play a role in onset of the disease. These findings imply that a HF diet affects levels of activated Caspase-3 in the brain, which is more evident with age, implying that age is most likely a contributing factor (Mhatre et al., 2014; Figure 16). The Caspase-3 activity in A β arc flies was only notable in the 10d flies compared to the wildtype NF group (Figure 16).

In general, age was not identified as a sole role player in neuronal apoptosis since it was not a factor that, on its own, was seen to affect Caspase-3 activity. The data also suggested that there are age-related changes in Caspase-3 only when the flies are fed a HF diet. This implied a HF diet was a more important role in Caspase-3 activation that happens to be worsened with age, rather than age itself playing a direct role in Caspase-3 activation in the brain. Therefore, this suggests that a HF diet induces more apoptosis in the *Drosophila* brain with age than a NF diet.

These results illustrated the possible interplay of genotype and diet on neuronal apoptosis in the brain, which is possibly worse in the hApoE4 flies. Caspase-3 cleavage sites have been detected and mapped in apolipoprotein E, which is the protein involved in this AD genotype, and therefore it is relevant that activated Caspase-3 was increased in the brains of 45d flies fed a HF diet (Khan et al., 2015). Additionally, the hApoE4 genotype mimics sporadic AD, in which genetics and environmental factors are involved in AD onset, and therefore it is not unlikely that AD pathogenesis in hApoE4 flies is more affected by a HF diet than A β arc flies (Feringa and van der Kant, 2021; Hampel et al., 2021).

3.4 Longevity Assay

3.4.1 Results

A longevity assay to observe diet-dependent effects on lifespan in three different genotypes, A β arc, hApoE4 and W₁₁₁₈, was performed at 18°C. Using a log rank test, it was determined that the differences in longevity between the three genotypes were statistically significant ($p < 0.0001$, Figure 17). All wildtype flies died before day 150

which was different to the survivability of A β arc and hApoE4 flies which lived to around 175 and 200 days respectively. It must be noted that lifespan data for A β arc female flies that were fed a HF diet was unexpected, as no other gender differences were seen, and while time did not allow it, future longevity assays should be repeated to determine if this result is significant. Using a log rank test, the survival for each genotype, within the two diet groups, illustrated that there were statistically significant differences between genotypes (HF $p = 0.0007$, NF, $p < 0.0001$, Figure 17).

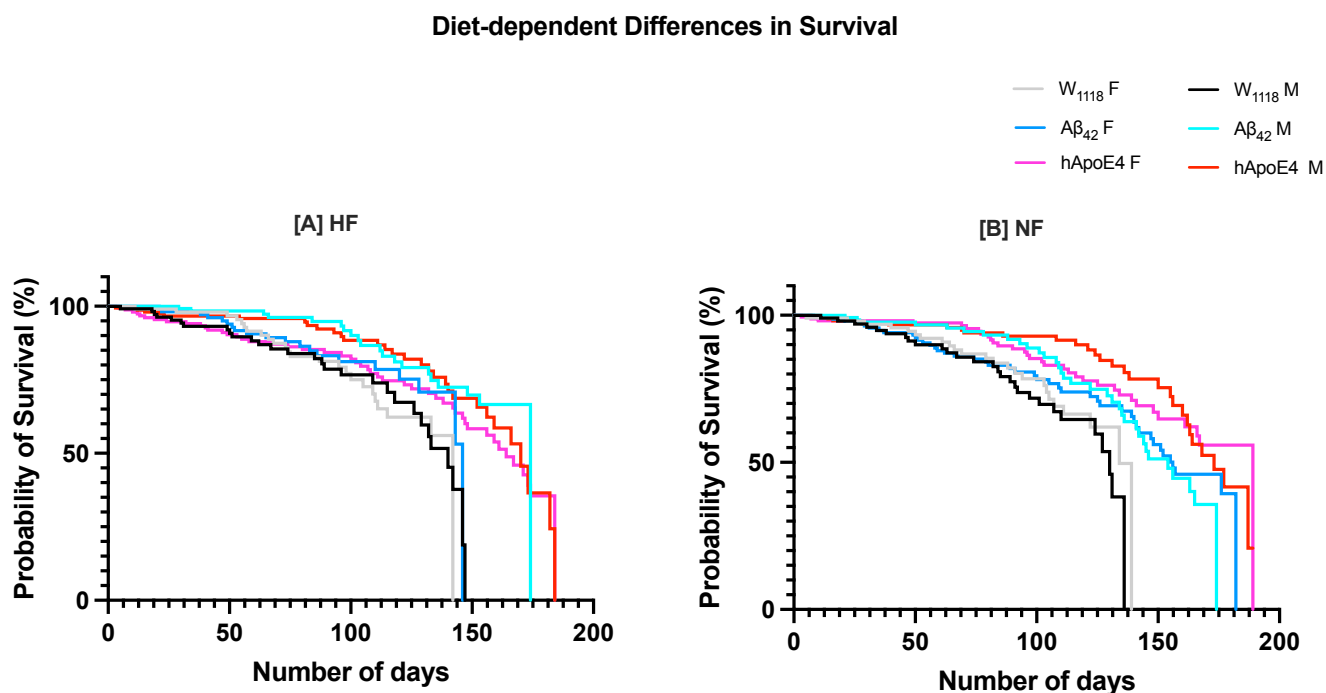


Figure 17: Longevity Curves by Diet and Genotype.

Data showed significant differences in survivability by genotype ($p < 0.0001$, log rank test). All conditions had a minimum of four different vials where $n \approx 30$ flies per vial. **[A]** Longevity curve for experimental conditions fed a HF diet. **[B]** Longevity curve for experimental conditions fed a NF diet.

Additionally, the longevity data was analysed by genotype to illustrate more directly diet-dependent effects on the lifespan of each genotype (Figure 18).

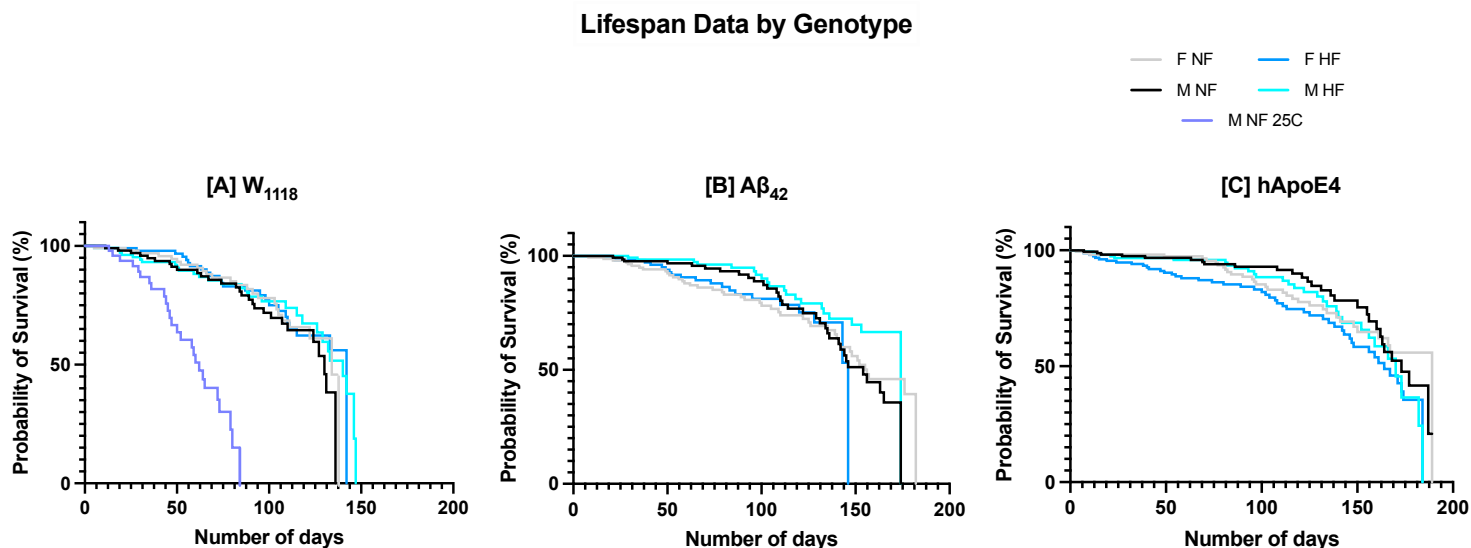


Figure 18: Longevity Curves by Genotype.

A] Longevity curve for male and female W_{1118} flies that were fed either a HF or NF diet. This curve also includes a past student's data on flies kept at 25 °C for reference. **[B]** Longevity curve for male and female $A\beta_{42}$ flies that were fed either a HF or NF diet. **[C]** Longevity curve for male and female hApoE4 flies that were fed either a HF or NF diet. Data showed significant differences in survivability by genotype ($p < 0.0001$, log rank test). All conditions had a minimum of four different vials where $n \approx 30$ flies per vial. This means ≈ 120 flies per experimental condition.

The median survival for each experimental group was plotted as this allowed for better comparison of the longevity results (Figure 19).

Median Survival by Diet and Genotype

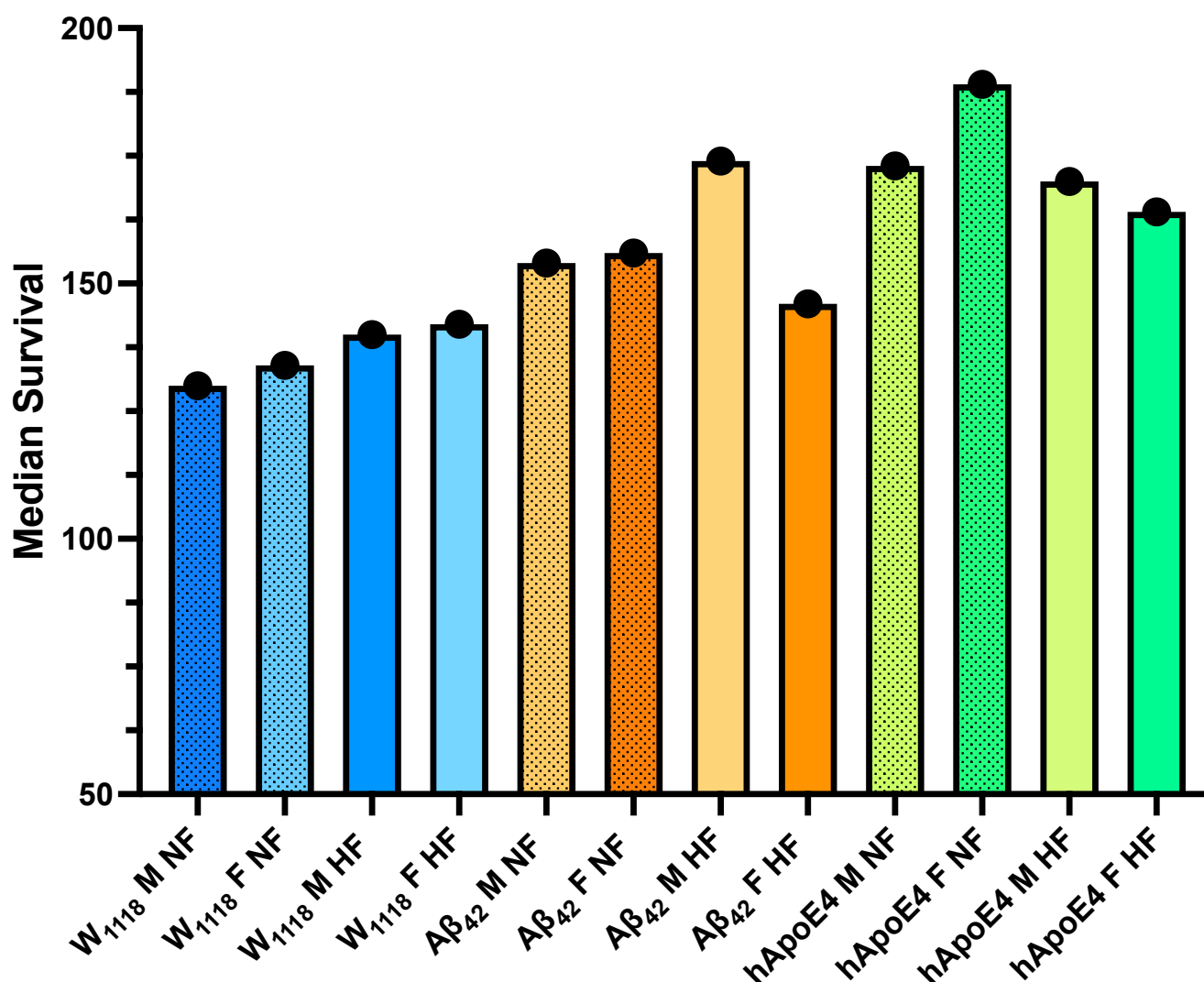


Figure 19: Median Survival.

The figure illustrates changing median survival for each condition at 18 °C as determined by a log-rank (Mantel-Cox) test.

3.4.2 Discussion

Longevity data was recorded to determine any diet-dependent effects on lifespan in three different genotypes, Aβarc, hApoE4 and W₁₁₁₈. Lifespans for all genotypes were longer than expected, regardless of whether the flies were fed a HF or NF diet. Since

this project involved novel research there were no longevity experiments reported on these mutants in the literature, conducted at the same temperature with a similar experimental design, with which to compare data and establish a “maximum lifespan”. Due to the length of the project, and unexpectedly long lifespans, it was not possible to repeat the longevity assay and so a “maximum lifespan” could not be established this way either.

One possible reason for the extended lifespan might be because all experimental conditions were kept at 18 °C which has been observed to increase average lifespan of different genotypes to around 165 days (Mhatre et al., 2014). In *Drosophila*, lowering body temperature is an effective mechanism used to extend longevity as cold temperatures have demonstrated a beneficial role in proteostasis and protein-aggregation prevention, which includes disease-related mutant proteins (Lee et al., 2023; Mhatre et al., 2014). Furthermore, lower temperatures are responsible for suppressing expression of human AD genes in *Drosophila* using the GAL4/UAS system (Mhatre et al., 2014). This is particularly important to consider in the AD model as the lower temperature at which the flies were kept could be what contributed to their extended longevity. This unexpected long lifespan will be investigated in future projects in the lab, however it is likely that there are various genetic factors that, together with a lower temperature, have contributed to the observed extended longevity.

Surprisingly, the two AD *Drosophila* groups, A β arc and hApoE4, lived longer than the wildtype flies (Figure 19). This was unexpected given that these genotypes are the AD experimental conditions and would therefore be expected to have shorter lifespans as they are diseased. These diseased genotypes possibly lived longer than the wildtype control because lower temperatures have been seen to prevent disease-related protein aggregation (Lee et al., 2023). In literature, GAL4 flies have been seen to have longer lifespans than flies crossed using the GAL4/UAS system and therefore GAL4 flies would most likely serve as a better control (Mhatre et al., 2014). Additionally, since AD pathogenesis involves A β aggregation, it is possible that the lifespan of the A β arc and hApoE4 flies were extended because there was less A β aggregation (Lee et al., 2023).

The A β arc and hApoE4 flies did however appear diseased, phenotypically, and therefore further investigation is required to determine the extent to which temperature affects protein aggregation and AD pathogenesis.

The longevity data showed the hApoE4 flies living longer than the A β arc flies which was expected as the hApoE4 genotype represents sporadic Alzheimer's and while this is a diseased line, it is possible that not all flies would exhibit AD pathogenesis in the same manner or severity since the onset of the disease is a combination of genetic and environmental factors.

In the W₁₁₁₈ flies, the lifespan of flies fed a HF diet and those fed a NF diet was similar, with the HF diet flies living slightly longer (Figure 18A). This was of interest as a HF diet was expected to shorten their lifespan, as seen in prior literature (Cormier et al., 2023; Trindade de Paula et al., 2016).

In the hApoE4 flies, there was virtually no difference in survival between the two different diets and between male and female. As seen in Figure 18C, all hApoE4 experimental groups were deceased around a similar number of days. This suggests that diet had little to no effect on lifespan which was unexpected as prior literature has observed decreased longevity in flies fed a HF diet (Trindade de Paula et al., 2016; Lee et al., 2020; Rivera et al., 2019)

The A β arc flies exhibited the greatest difference in lifespan between HF and NF conditions (Figure 18B). Flies that had been fed a NF diet were seen to live longer which followed the hypothesis that a HF diet would shorten lifespan (Trindade de Paula et al., 2016; Lee et al., 2020; Rivera et al., 2019). The female A β arc flies that were fed a HF diet were seen to live significantly shorter than the other A β arc experimental groups (Figure 18B). This was most likely an outlier as no other lifespan differences, in terms of diet or sex, between A β arc, hApoE4 and wildtype flies were observed.

These results did not indicate any obvious differences in longevity in flies fed a NF and HF diet which was unexpected as lifespan was expected to drop following exposure of flies to a HF diet, even only a 10 % coconut oil HF diet (Lee et al., 2020; Rivera et al., 2019; Trindade de Paula et al., 2016). There were no noticeable differences in lifespan between male and female flies in all experimental groups despite some literature showing decreased longevity in female flies fed a HF diet compare to male flies fed a HF diet (Baenas and Wagner, 2022). The condition which seemed to determine the biggest difference in lifespan was the genotype of the experimental group with A β arc and hApoE4 flies surviving significantly longer than W₁₁₁₈ (Figure 18). However, the effect of a low temperature on longevity cannot be overlooked as it could have been a factor which affected diet-, sex-, and genotype-related differences that may have been seen to affect longevity had the flies been kept at 25 °C (Mhatre et al., 2014).

3.5 Climbing Assay

3.5.1 Results

To investigate motor function in this *Drosophila melanogaster* AD model, we tested diet- and age-dependent differences in climbing ability of three different genotypes, A β arc, hApoE4 and W₁₁₁₈. A two-way ANOVA comparison of all climbing assay data revealed significant changes with age and genotype. Climbing ability was significantly altered in an age- and genotype-dependent manner (age: $F(11, 48) = 73.71$, $p < 0.0001$ and genotype: $F(1, 48) = 8.693$, $p = 0.0049$, Figure 20). For the W₁₁₁₈ flies, in both NF and HF diet groups, climbing ability was seen to worsen with age ($p < 0.05$, Figure 20).

Age-dependent Differences in Climbing

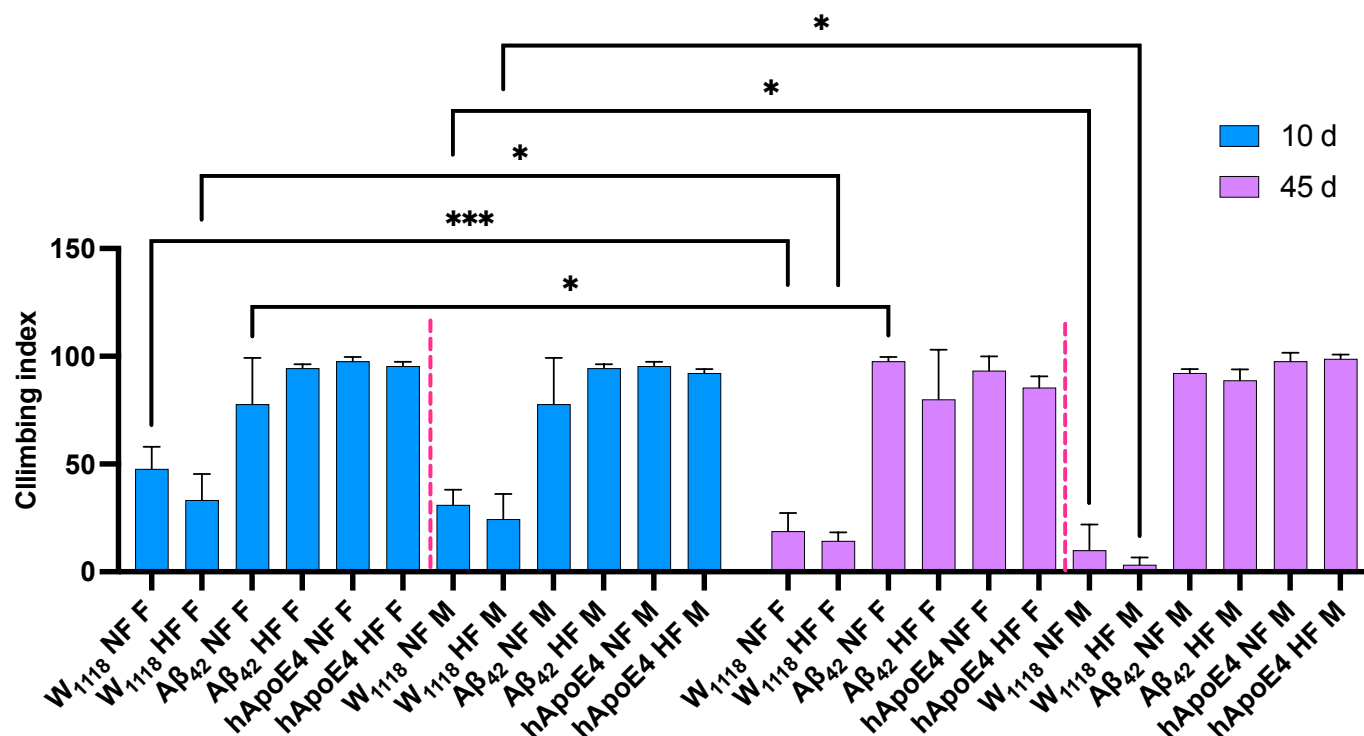


Figure 20: Climbing Assay Results for All Conditions.

A climbing assay was performed to assess motor function in three different genotypes which had been fed either a NF or HF diet. The assay was done at 10 or 45 days (d) of age. The data showed significant age- and genotype-dependent changes (age: $F(11, 48) = 73.71$, $p < 0.0001$ and genotype: $F(1, 48) = 8.693$, $p = 0.0049$, Two-way ANOVA, $n=10$ flies per vial). Significance is shown as * $p < 0.05$ and *** $p < 0.001$.

A two-way ANOVA comparison of climbing data for wildtype flies exhibited diet- and age-dependent changes that were statistically significant (diet: $F(3, 16) = 4.665$, $p = 0.0159$ and age: $F(1, 16) = 36.05$, $p < 0.0001$, Figure 21). The Aβ₄₂ and hApoE4 genotypes did not exhibit any diet-related trends in climbing, however there were some trends related to age.

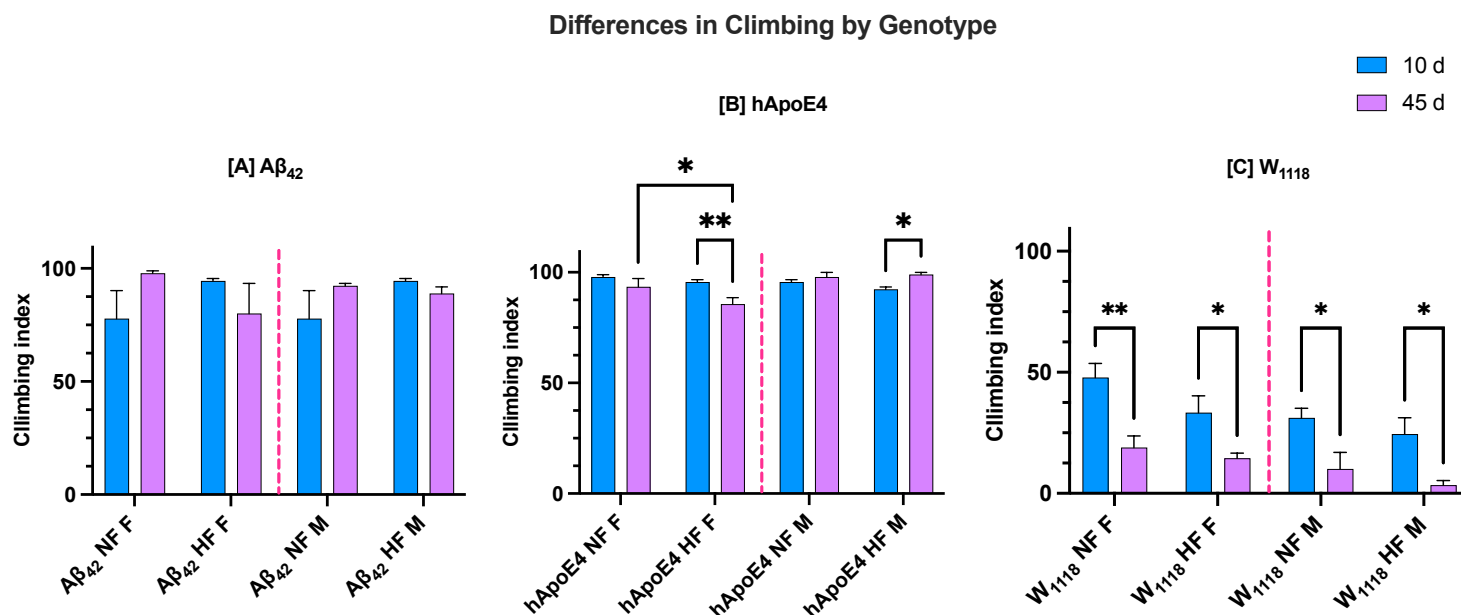


Figure 21: Climbing Assay Data by Genotype.

Motor function for each genotype was assessed, using a climbing assay, to determine the effects of age and diet. The assay was done, in each genotype, on flies at 10 or 45 days (d) of age that had been fed either a NF or HF diet. **A]** Climbing data for male and female Aβ₄₂ flies that were fed either a HF or NF diet. This curve also includes a past student's data on flies kept at 25 °C for reference. **[B]** Climbing data for male and female hApoE4 flies that were fed either a HF or NF diet. **[C]** Climbing data for male and female W₁₁₁₈ flies that were fed either a HF or NF diet. The data showed significant age- and genotype-dependent changes (diet: F (3, 16) = 4.665, p = 0.0159 and age: F (1, 16) = 36.05, p < 0.0001, Two-way ANOVA, n=10 flies per vial). Significance is shown as *p < 0.05 and **p < 0.01.

3.5.2 Discussion

To determine the effect of diet- and age-related changes on motor function, in three different *Drosophila* genotypes, a climbing assay was conducted on all experimental conditions. The data showed age- and genotype-related differences (Figure 20). It is important to note that the AD *Drosophila* displayed hyperactive behaviour and non-stop motion compared to that of the wildtype flies. This hyperactivity, which was seen in increase with age, is consistent with behaviour in AD studies of *Drosophila* and mouse models (Ping et al., 2015). The diet the flies were fed was not seen to influence differences in climbing ability or this hyperactive behaviour. This was not expected as previous literature has shown a HF diet affects climbing ability (Trindade de Paula et al.,

2016; Lee et al., 2020, Rivera et al., 2019). It is likely that the hyperactive behaviour observed in the AD experimental groups is the result of A β accumulation, as this accumulation promotes neuronal hyperactivity and is consistent with enhanced seizure activity in mouse models and AD patients (Ping et al., 2015). Whether this A β_{42} -induced hyperactivity is a measure of AD pathology is not yet known but it does play a role in age-dependent cognitive and motor decline in *Drosophila* AD models (Ping et al., 2015).

The AD genotype experimental groups, A β arc and hApoE4, showed a significant difference in climbing at 10d when compared to the wildtype flies (Figure 20). In 10d W₁₁₁₈, all exhibited climbing ability below 50 on the climbing index while the A β arc and hApoE4 groups had between 75-100 on the climbing index (Figure 20). For the 10d W₁₁₁₈ flies, a HF diet resulted in a reduced climbing index when compared to the 10d wildtype flies that were fed a normal diet (Figure 20). Conversely, the 10d HF A β arc flies showed better climbing ability than 10d NF A β arc flies (Figure 21A). This is most likely because of A β_{42} -induced hyperactivity, a characteristic of that genotype (Ping et al., 2015). There was no significant difference between the flies fed a HF and NF diet in the hApoE4 10d groups. The hyperactive behaviour observed in the 10d A β arc flies and the sedentary behaviour of the wildtype flies, were both exacerbated by a HF diet while the climbing ability of the hApoE4 10d flies appeared to be minimally affected by the HF diet.

In the 45d flies, the same pattern of climbing behaviour was observed across the different genotype groups and diets. The 45d W₁₁₁₈ flies exhibited much lower readings on the climbing index than the 45d A β arc and hApoE4 groups as well as the 10d W₁₁₁₈ experimental groups (Figure 20). This showed that climbing ability was affected by age, which was expected, and followed the same pattern of climbing behaviour observed between the different genotypes at 10 days old. The 45d wildtype flies that were fed a HF diet had a reduced climbing index when compared to the wildtype flies on a NF diet (Figure 21C). Similarly, the 45d HF A β arc flies showed reduced climbing ability when compared to 45d NF A β arc flies (Figure 21A). These results are expected as exposure

of *Drosophila* to a HF diet has been shown to decrease locomotor capacity by changes in geotaxis nature (Trindade de Paula et al., 2016; Lee et al., 2020; Rivera et al., 2019).

There did not appear to be any major differences between the climbing ability of the hApoE4 *Drosophila*, in both NF and HF groups, where there was only a slight reduction in climbing ability for the 45d hApoE4 flies compared to 10d hApoE4 flies, most likely the result of natural ageing (Figure 21B). In female hApoE4 flies, climbing ability worsened with age, in both HF and NF groups, and was seen to improve with in male hApoE4, in both HF and NF groups. This difference was not focused on as there were no other sex differences in climbing ability for any other experimental conditions.

Generally, at 45d, the A β arc flies had similar climbing indexes and there was a less noticeable difference between HF and NF diets than there was in the 10d A β arc flies' conditions (Figure 20). This is possibly because in the 45d A β arc flies, A β accumulation was occurring regardless of the diet they were being fed. Both NF and HF experimental groups would have been hyperactive and had A β accumulation and therefore exhibited increased climbing ability regardless of diet (Ping et al., 2015).

For the W₁₁₁₈ flies, there was a difference between the age groups as well between the two diets (Figure 21C). As expected, there was a difference in climbing ability between the 10d and 45d wildtype flies which was most likely a natural effect of ageing on motor function (Trindade de Paula et al., 2016; Lee et al., 2020, Rivera et al., 2019, Figure 21C). For both 10d and 45d groups, HF W₁₁₁₈ flies had decreased climbing ability compared to NF W₁₁₁₈ flies (Figure 21C). This can be attributed to a HF diet impairing mobility in the negative geotaxis test, as outlined in Trindade de Paula et al., (2016).

4 General Discussion:

This study set out to investigate the effects of a high-fat diet on oxidative stress and neurodegeneration, as part of AD pathology, in *Drosophila* expressing different genes to predispose and develop AD. To determine the effects of diet on AD pathogenesis and onset, biomarkers of oxidative stress and AD pathogenesis, longevity, and locomotor activity were assessed. Important differences were found in redox stress levels, cell death signalling, locomotion, and survival.

The SOD assay showed significant age- and genotype-dependent differences with reduced SOD levels in aged flies and AD genotypes, hApoE4 and A β arc, when compared to control flies (Figures 10 and 11). It was expected that SOD activity would increase in these diseased genotypes as a compensatory response to oxidative stress (Ali et al., 2019; Kizhakke P. et al., 2019; Trindade de Paula et al., 2016). The diet-related changes did not show any obvious patterns which meant it was not possible to make a definitive conclusion on the effects of diet on SOD activity. The effect of a HF diet on oxidative stress and SOD activity is something that will need to be explored in further research. The lack of diet-dependent changes in the SOD assay results was similar to that Trindade de Paula et al. (2016) where there was no effect on SOD activity in flies fed a 10% HF diet.

The lipid peroxidation (MDA) assay also showed some differences however these were most noticeable in W₁₁₁₈ and hApoE4 flies. Interestingly, MDA was seen to decrease with age in wildtype flies which was not expected as oxidative stress, and consequently lipid peroxidation, increases with age (Ali et al., 2019; Kizhakke P. et al., 2019; Trindade de Paula et al., 2016; Figure 12A). This expected result occurred in the hApoE4 flies where MDA levels increased with age (Figure 12C). In 45d male W₁₁₁₈ and hApoE4 flies, MDA levels increased in those fed a HF diet compared to those fed a NF diet (Figure 12A & 12C) which was expected as oxidative stress causes cell damage processes to occur, thereby increasing MDA levels as a result of lipid peroxidation (Trindade de Paula et al., 2016). Interestingly, the lipid peroxidation assay also

displayed some sex-related differences with MDA decreasing in 45d female conditions for both W_{1118} HF and hApoE4 NF groups (Figure 12A & 12C). These results were significant as they were exceptions to noticeable trends in wildtype and hApoE4 conditions. This is interesting as prior literature has shown sex-related differences, especially where dietary modifications are concerned (Baenas and Wagner, 2022). Female flies have been observed as being more sensitive to HF diets as a result of adaptation to reproductive functions (Baenas and Wagner, 2022). While sex-related differences were not the focus of this project it does prompt an intriguing inquiry into how gender might impact response to these mutations and diets.

Immunohistochemistry (IHC) showed significant genotype-, age- and diet-related differences in activated Caspase-3 in the *Drosophila* brain. Activated Caspase-3 increased with age and in a HF diet, and was also seen to be higher in AD genotypes (Figure 16). Most importantly, age alone did not emerge as a sole contributor to neuronal apoptosis, as it did not appear to influence Caspase-3 activity independently. Rather, the results suggest that age-related increases in Caspase-3 were only noticeable in conditions in which flies had been fed a HF diet. Since Caspase-3 is a known key mediator of neuronal apoptosis, it could be said that a HF diet is a factor affecting apoptosis in the brain (Fan and Bergmann, 2010; Khan et al., 2015). However, these results are baseline and further investigation will be needed in order for more conclusive evidence.

The longevity assay displayed extended lifespans for all genotypes which was largely attributed to the maintenance of all experimental conditions at 18 °C (Mhatre et al., 2014). In Trindade de Paula et al. (2016), flies fed a 10% and 20% HF diet showed significantly reduced lifespan, however these flies were kept at 25 °C. In this project, it was not possible to keep the flies at 25 °C, but these differences in results indicates a possible significant role of temperature on lifespan. This is particularly noteworthy in an AD model as reduction in body temperature increases longevity and also has been seen to play a beneficial role in preserving proteostasis and preventing protein aggregation (Lee et al., 2023, 2020; Mhatre et al., 2014; Rivera et al., 2019; Trindade de Paula et

al., 2016). There were no obvious diet-dependent differences apart from NF A β arc flies living longer than HF A β arc flies (Figure 18B). These findings do not reveal any apparent diet-dependent variations in lifespan which was unexpected as a HF diet has been shown to shorten longevity (Lee et al., 2020; Rivera et al., 2019a; Trindade de Paula et al., 2016). The factor that appeared to exert the most influence on lifespan was genotype, with the AD genotype flies living significantly longer than the wild type flies (Figure 19). This could be the GAL4 driver line interplay in the GAL4/UAS system as other AD genotypes, generated using this system, were seen to have longer lifespans, especially at 18 °C (Mhatre et al., 2014). However, it is important that that potential impact of lower temperatures on longevity is not disregarded as it is not fully understood to what extent temperature could have influenced diet-, sex-, and genotype-related differences in the longevity results (Mhatre et al., 2014).

The climbing assay data, to assess motor function, showed age- and genotype-dependent differences (Figure 20). The W₁₁₁₈ flies, regardless of diet, exhibited a decline in climbing ability with age, which is consistent with the natural ageing process (Mhatre et al., 2014; Trindade de Paula et al., 2016). Additionally, diet affected climbing ability of W₁₁₁₈ flies, with a decline in those fed a HF diet (Figure 21C). This was anticipated as Trindade de Paula et al. (2016) discussed the significant deleterious impact of a 10% and 20% HF diet on locomotion. However, diet did not seem to have any significant effects in the AD genotypes. This is interesting as a HF diet impacted climbing in W₁₁₁₈ flies and therefore this suggests a need for further investigation into the effects of a HF diet on climbing in AD genotypes. Additionally, there were some age-related trends displayed in the AD genotypes and these age-related changes were less pronounced than in the wildtype flies (Mhatre et al., 2014; Trindade de Paula et al., 2016, Figure 20). It is important to note that the hyperactivity observed in the A β arc and hApoE4 flies could potentially mask diet- and age-related decline in motor function (Ping et al., 2015). Additionally, in future studies it will be important to consider sex-related differences as prior literature has shown climbing ability being significantly more affected female flies fed a HF diet than in male flies fed a HF diet (Baenas and Wagner, 2022). While this was not observed in our data, it is something to consider and is

relevant given sex-specific differences in octopaminergic neuron activity which is involved in generating different endurance responses in flies (Baenas and Wagner, 2022).

It is worth noting that coconut oil was used in the HF diet, as coconut oil has demonstrated potential neuroprotective effects in rat models, improving memory and cognitive symptoms in AD (Mirzaei et al., 2018). This raises the intriguing possibility that the HF diet employed in this research project may have had a neuroprotective effect instead of the anticipated exacerbation of AD pathogenesis. Although we didn't observe any definitive evidence of these neuroprotective effects in this project, it presents a thought-provoking concept for future investigations into the effect of a high-fat diet on AD pathogenesis.

Lastly, it is vital to acknowledge the effects of lower temperature on *Drosophila* models of human disease. Lower temperatures suppress expression of human AD genes, since the GAL4 protein is minimal at 16 °C, and therefore AD pathogenesis is not exhibited to full extent in A β arc and hApoE4 flies (Mhatre et al., 2014). Additionally, lower temperatures have been observed to affect results in other assays which is important to consider (Mhatre et al., 2014). It is crucial that that potential impact of lower temperatures, which was necessary to maintain solidity of the HF food, is not disregarded as it is not fully understood to what extent temperature could have influenced differences related to diet, sex, and genotype (Mhatre et al., 2014).

5 Future Work:

Future iterations of this projects should focus on increasing accuracy of results which will facilitate the extraction of meaningful trends and inferences. For instance, due to time constraints, biochemical assays were not repeated which affects the reliability of the results, especially when interpreting unexpected findings. Additionally, to ensure the validity of results, both NF and HF vials should be stored horizontally with the same amount of food and population density of flies.

Further research avenues could explore a more 'unhealthy' HF diet with the inclusion of a diet rich in 'unhealthy' fats, such as saturated and trans fats. Given the potential neuroprotective benefits of coconut oil in the diet (Mirzaei et al., 2018), it would be valuable to repeat this research project including diets of 20 % and 30 % coconut oil as this could yield valuable insights (Trindade de Paula et al., 2016). Additionally, it would be interesting to examine the impact of synbiotics on AD symptoms and pathogenesis (Westfall et al., 2019). The application of synbiotics presents a possible therapeutic target, given their observed effects in increasing survivability and motility and rescuing of amyloid beta deposition which could further our understanding of the role of the gut-brain axis in AD (Westfall et al., 2019).

The hyperactive behaviour observed by *Drosophila* in the AD genotype groups warrants further investigation. The hyperactivity observed in the A β arc and hApoE4 flies could have potentially masked the diet- and age-dependent differences in climbing ability (Ping et al., 2015). Additionally, considering the hApoE4 and A β arc fly lines are generated using the UAS-GAL4 system, including GAL4 as a control would likely be more relevant.

Repeating the project with older experimental groups would be beneficial. While the flies at 45d displayed signs of disease, more pronounced indications of AD pathogenesis became evident past 60-70 days in the longevity assay. Given the extended lifespans, as shown in the lifespan assay, it is reasonable to assume the flies would only be considered "aged" past the mid-lifespan mark of around 90 days.

Lastly, it would be beneficial to repeat this project with more of a focus on the expression of human AD genes in *Drosophila melanogaster* at different temperatures. Prior literature, and our results, indicate that lower temperature does most likely play a role on expression of human AD genes (Mhatre et al., 2014). This is because the expression of these AD UAS transgenes is affected as a result of the activity of the GAL4 protein being affected (Mhatre et al., 2014). Therefore, the effect of lower

temperatures on expression of AD genes in *Drosophila* and AD pathogenesis in *Drosophila* disease models needs to be better understood.

6 Conclusion:

This project aimed to investigate the effects of a high-fat diet on oxidative stress and neurodegeneration in an AD model in *Drosophila melanogaster*. SOD levels decreased in AD genotypes with age, especially when they were exposed to a HF diet, suggesting an impaired oxidative stress response or less overall protein as there was increased apoptosis in the brain (Figure 14 and Figure 15).

The MDA assay showed complex age- and diet-related variations with MDA levels increasing with age and a HF diet in hApoE4 flies while the opposite occurred in W₁₁₁₈ flies. This assay also yielded interesting results regarding female flies fed a NF diet, which presents a compelling avenue for further research. Notably, activated Caspase-3 in the *Drosophila* brain showed age-, genotype- and diet-dependent increases, indicating a role in neuronal apoptosis. Longevity was extended significantly at 18 °C with genotype having the most significant influence on lifespan and no clear diet-dependent variations. The climbing assay showed age-related declines in motor function, particularly in W₁₁₁₈ flies. There were also genotype-dependent differences, with the AD genotypes showing hyperactivity, a factor which is interesting in itself and could have potentially masked other factors affecting climbing ability.

Expression of A β arc and hApoE4 genes will need to be tested since temperature effects were observed. It is most likely that expression of A β arc and hApoE4 UAS transgenes are affected by temperature, due to the activity of the GAL4 protein being affected. The effects of temperature on gene expression will be important for analysing results in future work.

Interestingly, the use of coconut oil in the HF diet hinted at potential neuroprotective effects, challenging the expected exacerbation of AD pathogenesis. These findings collectively suggest that a HF diet, age, and genotype may influence oxidative stress

and the response to oxidative stress, as well as AD pathogenesis. To conclude, the findings of this study revealed compelling insights that warrant further investigation into the relationship between diet, oxidative stress, and neurodegeneration in AD pathogenesis.

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