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Alzheimer's Disease: Investigating the neuroprotective role of Polyunsaturated fatty acids against β-Amyloid accumulation using SH-SY5Y cell models

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Abbreviations

µl Microlitre

µM Micrometre

7TM Seven-transmembrane domain receptor

ACh Acetylcholine

AD Alzheimer's Disease

AIF1 Allograft factor 1

ALA Alpha-linolenic acid

APH-1 Anterior pharynx-defective 1

APOE4 Apolipoprotein E4

APP Amyloid precursor protein

APPase Amyloid precursor protein enzyme

APPswe Swedish mutated amyloid precursor protein

ATCC American type culture collection

Aβ Beta Amyloid

BACE1 Beta-site APP cleaving enzyme 1

BDNF Brain-derived neurotrophic factor

C=C Double carbon bonds

CH3 Methyl group

CHAT Choline acetyl transferase enzyme

CI	Cholinesterase inhibitors
CO2	Carbon dioxide
CSF	Cerebrospinal fluid
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DSM-V	Diagnostic and Statistical Manual of Mental Disorders 5
eFAD	Early onset familial Alzheimer’s disease
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FAD	Familial Alzheimer’s disease
GPR40	G-protein coupled receptor 40
ISF	Interstitial fluid
MMSE	Mini-Mental Sate Exam
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NCT	Nicastrin
NFT	Neurofibrillary tangles
NHS	National health service
PBS	Phospahte-buffered saline
PEN-2	Presenilin enhancer protein 2

PET Positron emission tomography

PI 3-K Phosphatidylinositol 3-Kinase

PS Presenilin

PSEN1 Presenilin 1

PSEN2 Presenilin 2

PUFA Poly-unsaturated fatty acid

RA Retinoic acid

R-OOH Carboxyl terminal

RXR Retinoid-X-Receptor

SPECT Single photon emission computed tomography

T75 T-flask 75cm

UK United Kingdom

Abstract

Alzheimer's disease is the most common type of dementia characterised by cognitive impairments such as memory loss as well as behavioural symptoms like depression and aggression. The hallmark of the disease is the accumulation of beta-Amyloid (Aβ) as well as the presence of neurofibrillary tangles (NFT) caused by the hyperphosphorylation of tau protein. Current treatment methods target the symptoms experienced in AD rather than the degeneration itself, this research was conducted to find a method that could have the potential to slow down the progression of the disease. It is well known that Omega-3 plays a vital role in brain health, specifically DHA (docosahexaenoic acid) which expresses neuroprotective properties. SHSY5Y cells were used to model AD neurones, they were treated with various concentrations of different polyunsaturated fatty acids (PUFA), including DHA. Cell viability, as well as the volume of Aβ, was measured and compared against control cells (native) to display the potential for PUFAs to be used as a viable treatment method for AD. It was found that cells treated with DHA concentrations of 1µM and 10µM showed a significant increase in cell viability as well as a decrease in levels of Aβ42 accumulation compared to the control and other PUFA treatment groups.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most prevalent subtype of dementia; affecting between 50-75% of the dementia population (Duong, Patel and Chang, 2017). According to the Alzheimer's society, there are currently, 850,000 cases of Dementia in the UK and between 50-75% of these cases are Alzheimer's disease. This figure is believed to increase to 1.6 million by 2040 (Alzheimer's Society). The Office for National Statistics showed that in 2015, 61,686 people died from Alzheimer's disease - over taking the mortality figures for heart disease and stroke, making it the leading cause of death in England and Wales, and more recent research from 2019 also supports this claim (Walton, 2019). AD is characterised by cognitive and behavioural changes. Cognitive symptoms include memory loss, apathy, sleep disruption and depression, the behavioural symptoms often affect everyday life due to the gradual cognitive decline leaving people often unable to complete simple everyday tasks and look after their basic needs (Li et al., 2014). Although there is no known cause, it has been concluded that there is a genetic predisposition for the disease, where familial AD (FAD) accounts for up to 6% of all AD cases, as well as environmental risk factors contributing to the pathogenesis of the disease. The main hallmarks of AD are; an accumulation of amyloid-β plaque in the brain as well as neurofibrillary tangles, vascular damage due to the excessive concentration of plaque, and neuronal cell loss and death (Hardy and Higgins., 1992).

Average Lifespan and diagnosis

The average age of onset of the disease is 65, which is when the first symptoms may appear. However, 5-6% of individuals develop early-onset AD, which emerges between ages 30 and 60 (National Institute on Aging). The life expectancy for people with AD varies, however, the average

life expectancy after diagnosis is 8-10 years. In some cases, this can be as low as 3 years and as high as 20 years (Holland, 2016). It has been determined that the disease begins in the brain at least 20-30 years before the emergence of the first symptoms such as memory impairment. By the time an individual has been diagnosed with the disease, the level of damage in the brain is thought to be irreversible (Selkoe and Hardy, 2016).

The diagnosis of the disease is based on the criteria in the Diagnostic and Statistical Manual of Mental disorders, fifth edition (DSM-5), where the disease is split into two categories; Mild neurocognitive impairment and Major neurocognitive impairment. The mild neurocognitive category consists of, mild cognitive decline In memory and learning, and gradual and progressive decline in cognition. The major neurocognitive category includes progressive cognitive decline, severe enough to interfere with daily life and activities, which is not caused by delirium or other neurological or psychiatric disorders (Apostolova, 2016). The criteria for the diagnosis of Alzheimer's or Dementia no longer require the existence of memory impairment for an individual to be diagnosed, which was essential in previous versions of the DSM. The Mini-Mental State Exam (MMSE) is a widely used tool to determine the level of cognitive decline present in an individual and contributes to the accurate diagnosis of the disorder (Arevalo-Rodriguez et al., 2015). Since the release of the DSM-V, great progress has been made in the methods of identifying the changes occurring in the brain, from neuroimaging techniques to the use of biomarkers. Neuroimaging techniques such as PET, SPECT (single-photon emission computed tomography) and MRI can be used to aid in the detection of the disease. The validity of Amyloid-PET imaging has proved to be highly specific in detecting moderate to severe levels of amyloid accumulation in the brain. The most widely used cerebrospinal fluid (CSF) biomarkers measure the levels of Aβ 42 (responsible for amyloid plaques found in the brain), tau, and phosphorylated tau (forming the tau neurofibrillary tangles in the brain) these three components are described in further detail in 'Pathogenesis' section. The levels of Aβ 42 in the CSF will be low in value in individuals with Alzheimer's disease and the levels of Tau and phosphorylated tau will be high. Although these practices are used in

some clinical settings, they aren't used for all cases, considering their effectiveness in the early detection of the disease, as well as the level of degeneration that has occurred (Biomarkers for Dementia Detection and Research, 2021).

Cost to NHS

The total cost of care for those with dementia in the UK is £34.7 billion. These costs are split into three categories: Healthcare costs (costs to the NHS), Social Care costs (residential care or homecare) and unpaid care (being cared for by family members).

Social care has the largest proportion of the total cost, at 45%, valued at £13.9 billion a year; £8.3 billion is paid by family members, and the remaining £5.6 billion is paid by the state. The average cost of homecare for 1 person with Alzheimer's/dementia can cost a family around £38,000 a year and £100,000 a year for residential care. It is clear that Alzheimer's disease is not only a great burden on family members emotionally, but also financially just to keep their loved ones properly cared for. On top of this, it is also a large burden on the NHS and the state, and it is predicted to sharply increase over the next two decades to £94.1 billion a year. Alzheimer's has such great annual costs, however very little is spent on the research of Alzheimer's and Dementia each year, and it is clear that there needs to be an increase in funding and research in this field (Alzheimer's society, 2018).

Symptoms of Alzheimer's Disease

Alzheimer's disease is characterised by both cognitive and behavioural symptoms; however, no individual's experience of Alzheimer's is the same due to the complexity of the disease and the variety of symptoms that can be experienced, depending on which regions of the brain are deteriorating. Generally, symptoms will start mild and increase in severity over time as the

disease progresses. Usually, the first sign of AD is memory loss; individuals will have trouble recalling past events and learning new information due to damage in the hippocampus, responsible for learning and memory. Many individuals experience changes in mood and behaviour such as increased anxiety, aggression, and the development of depression. On top of this, some may suffer from hallucinations, delusions, and paranoia. As the cognitive impairments progress, it can prevent the individual from completing daily tasks, and things that follow a routine such as brushing teeth and getting dressed. They commonly experience problems with language (i.e. speech and reading) organising their thoughts and recognising family members. Individuals gradually become reliant on a carer as the disease progresses from the early-moderate-late stage.

Disturbances experienced with orientation cause individuals to become confused, they may get lost, lose track of the days and have trouble with the perception of time. This is due to degeneration in the Entorhinal cortex (National Institute on Aging, 2017) located in the ventromedial temporal lobe, it contains axons which connect the hippocampus to the neocortex; responsible for higher-order thinking and functions (van Hoesen, Hyman and Damasio, 1991). It is a region that is found to have a high level of atrophy in individuals with Alzheimer's disease.

The disease progresses in three stages; Mild (early-stage), Moderate and Severe (late-stage) Alzheimer's, when late-stage AD is reached, symptoms can be very severe and linked to the death of the individual. Severe/ late-stage Alzheimer's is highly debilitating, at this stage individuals are fully dependant on their carers, they are unable to communicate and usually are on permanent bed rest. Symptoms experienced are seizures; dysphagia (difficulty swallowing) leading to weight loss, as well as, skin infections; and loss of bowel and bladder control (National Institute on Ageing, 2017).

Alzheimer's disease is not always the cause of death for those who live with it, as some may die from other health conditions or natural causes. However, patients with late-stage Alzheimer's

often develop two medical conditions, Dysphagia and Aspiration pneumonia, these are the most serious medical conditions experienced in late-stage Alzheimer's. Dysphagia, problems with swallowing or the inability to swallow at all has proved severe as it not only causes weight loss in these patients but prevents them from receiving required nutrients from food. Research suggests that aspiration pneumonia is the most common cause of death for those with late-stage Alzheimer's. It is caused by the inhalation of stomach contents, food or liquids into the lungs due to the loss of the gag reflex, dysphagia and the reduced level of consciousness experienced by patients (Kalia, 2003).

It is thought that changes in the brain occur up to two decades before the onset of cognitive symptoms in Alzheimer's. The accumulation of Amyloid-β protein in the brain, over time, builds up and aggregates forming senile amyloid plaques responsible for the degeneration of the brain and its' functions (Alzheimer's society, 2019). According to neurologists, by the time people show symptoms such as memory impairment, their brains have already become severely degraded, "that no type of therapy will fully heal them" (Schindler et al., 2019). Therefore, early intervention is a crucial step in treating patients with the disease, it would allow early treatment which could potentially help alleviate symptoms in the earliest stages, in hopes of individuals maintaining a good quality of life for longer. Even though the test will not be 100% accurate in predicting who will or will not develop Alzheimer's disease, it would be able to identify individuals/groups who are more susceptible (Alzheimer's society, 2015).

Research at Washington University School of Medicine has formulated a blood test allowing them to measure Amyloid-β protein levels present in the blood. Using these results they can predict whether Amyloid-β has accumulated in the brain. To improve the accuracy of the test, they combine the blood Amyloid-β levels with the age of the individual and the percentage of genetic risk based on the presence of the APOE4 allele (two main risk factors for AD). From this, they were able to identify those with early Alzheimer's related brain changes with 94% accuracy. It was

found that this blood-test method is more sensitive than a PET brain scan at detecting deposits of amyloid-β in the brain (Schindler et al., 2019). However, this is new research and requires further testing, it has been tested on a small sample of people over 50 (158 participants) and increasing it to a wider scale could prove beneficial.

However, testing for preventative treatments can be a long and expensive process, requiring specialist screening equipment and thousands of participants who are healthy and those with a build-up of amyloid, but do not show cognitive symptoms yet (important to be before they show any symptoms) finding participants who match the criteria may be difficult (Schindler et al., 2019).

Amyloid Precursor Protein (APP) Processing

The amyloid precursor protein (APP) is a plasma membrane protein naturally existing in healthy brains; responsible for neural growth and repair. It is synthesised in the endoplasmic reticulum and translocated via the Golgi apparatus to the plasma membrane. APP is normally broken down by enzyme APPase, then drained and eliminated via the interstitial fluid pathway (Weller, Carare and Boche, 2017) the purpose of this mechanism is to prevent the accumulation of amyloid- β and thus subsequent neurodegeneration (Yin et al., 2017).

APP can be broken down via two pathways The non-amyloidogenic pathway and the amyloidogenic pathway, the main difference between the two pathways is that one results in the neurotoxic form of Aβ.

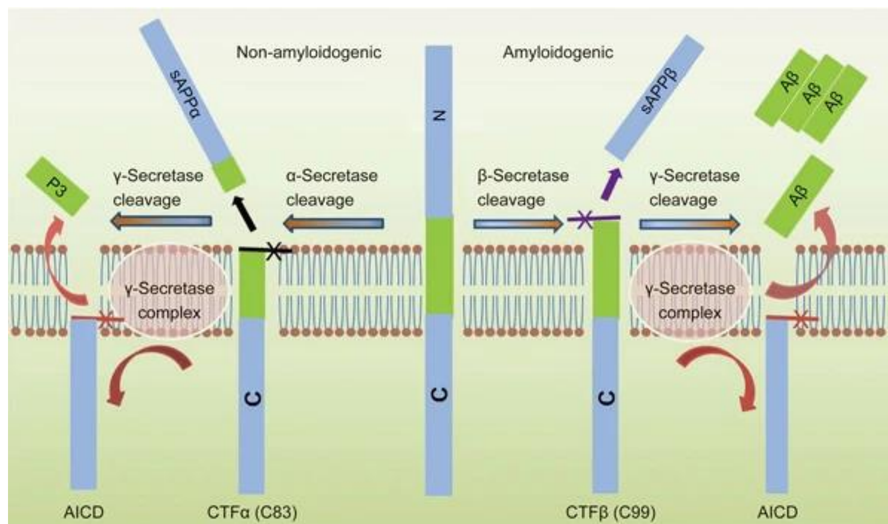


Figure 1. A diagram of APP processing; both the amyloidogenic and the non-amyloidogenic pathway and the enzymes involved are shown above (Chen et al., 2017).

The processing of APP involves three enzymes; Alpha (α)-secretase, Beta (β) -secretase and Gamma (γ)-secretase. α-secretase is an enzyme which comes under the family of α-disintegrin and metalloprotease domain (ADAM) (Lichtenthaler., 2012) it cleaves APP within the Aβ chain, thus preventing the formation of neurotoxic Aβ (Postina., 2008). β-secretase also known as beta-site APP cleaving enzyme 1 (BACE1) is a transmembrane protein part of the amyloidogenic pathway as it is an enzyme known to lead to the initiation of Aβ production due to the site at which it cleaves APP (Vassar et al., 2009). γ-secretase is a multi-protein complex constituting of four main proteins; presenilin (PS), Nicastrin (NCT), anterior pharynx-defective-1 (APH-1) and presenilin enhancer protein 2 (PEN-2) (Wolfe, 2008; Zhao and Zhao, 2013). It is involved both the amyloidogenic and non-amyloidogenic pathways. The majority of APP is processed using the non-amyloidogenic pathway over the amyloidogenic pathway (Zhao et al., 2020).

The non-amyloidogenic pathway involves α- secretase and γ-secretase, both enzymes sequentially cleave APP at different regions, creating smaller soluble fragments, that are readily broken down and disposed of by the body (Zhang et al., 2012). The α-secretase cleavage site is within the Aβ chain between Lys16 and Leu17, when cleaved it forms an amino (N) terminal fragment called secreted APP (sAPP) and the carboxy-terminal fragment known as CTFα (C83). Following this, CTFα isβ cleaved by γ-

secretase forming p3 (an extracellular peptide) and AICD (APP intracellular domain) thus preventing the formation of Aβ (Zhao et al., 2020).

The Amyloidogenic pathway involves β- secretase (BACE1) and γ-secretase. This pathway produces the neurotoxic Aβ due to the cleavage sites of APP. β-secretase cleaves APP, producing CTFβ (C99) and the N-terminal sAPPβ. CTFβ is further cleaved by γ- secretase, resulting in the release of extracellular Aβ peptides and AICD (Chow et al., 2011). Several peptides of varying lengths can be generated from the cleavages by β and γ – secretase, ranging from Aβ-38 to Aβ-42 (Zhao et al., 2020). Aβ-40 and Aβ-42 are the two most common forms of Aβ found in the brain, Aβ42 is more toxic than the more abundantly produced Aβ 40, due its' tendency to aggregate faster than Aβ-40 (Gu & Guo, 2013).

Beta-Amyloid (Aβ)

It is known that the amyloid precursor protein (APP) is a plasma membrane protein naturally existing in healthy brains; responsible for neural growth and repair. It is synthesised in the endoplasmic reticulum and translocated via the Golgi apparatus to the plasma membrane. APP is normally broken down by the enzyme APPase, then drained and eliminated via the interstitial fluid pathway (Weller, Carare and Boche, 2017) the purpose of this mechanism is to prevent the accumulation of amyloid- β and thus subsequent neurodegeneration.

The processing of APP results in two common isoforms of Aβ; Aβ-40 and Aβ-42, in which both exist in soluble and insoluble forms (Karelina et al., 2017). Aβ monomers can aggregate into various forms; amyloid fibrils, protofibrils and oligomers, the amyloid oligomers are small, soluble structures which allow them to spread across the brain as well as be eliminated. In contrast, the amyloid fibrils are larger, insoluble structures which can aggregate further to form the insoluble Aβ plaques (Chen et al., 2017). Over time, these aggregates and plaques lead to the formation of senile plaques which are thought to influence synaptic dysfunction and nerve cell death. As these cells die, the affected brain regions begin to deteriorate, therefore preventing signals from being transmitted efficiently or at all,

thus leading to the main symptoms observed in AD and progressive cognitive decline (Carter & Lippa, 2001).

It was previously believed that high levels of insoluble Aβ40 and Aβ42 were the cause of dementia, due to them being highly neurotoxic. However, more recently there has been evidence highlighting a strong link between the presence of soluble Aβ and cognitive impairment and synaptic death viewed in those with AD (Prins and Scheltens, 2013; Li et al., 2014). In eFAD, a mutation in specific genes can cause high levels of Aβ 42, which leads to Aβ accumulation and plaques (Thinakran, 1999). Research has shown that the pre-fibrillar forms of soluble Aβ are more neurotoxic than the insoluble forms as when hippocampal neurones were exposed to Aβ, synapse loss and dysfunction were induced (Shrestha et al., 2006).

However, there has been plenty of research which has contradicted this theory, as research by Wang et al (1999) examined the levels of Aβ40 and Aβ42 in healthy, pathologically ageing and AD brains. It was concluded that the healthy brains contained the highest levels of Aβ40 (50%) and Aβ42 (23%), followed by the pathologically ageing group (Aβ40-8% and Aβ42- 0.7%). The AD brains contained the lowest levels of Aβ40 (27%) and Aβ42 (0.7%) which shows that in increase in Aβ does not equate to cognitive decline and neurone death experienced with AD. Further research has shown that the main difference in levels of Aβ between healthy and AD brains is the switch from higher levels of soluble Aβ40 and Aβ42 to increased levels of insoluble Aβ40 and Aβ42, as well as a significant overall increase in the volume of Aβ40. Those with AD showed lower levels of soluble Aβ42 than those with normal cognition (Zaretsky et al., 2022). Therefore allowing us to believe that cognitive decline experienced in AD is due to the decrease in soluble forms of Aβ rather than an increase in the insoluble forms of Aβ, which refutes a lot of previous research in AD, supporting this was research by Sturchio et al (2021).

Supporting this a drug called Lecanemab was developed as a treatment for AD as it targets and eliminates insoluble forms of Aβ, during its pre-clinical stage it was tested on mice models of AD,

where it reduced levels of insoluble Aβ in the brain and CSF (Tucker et al., 2015). Following this, in 2018 further mice studies were conducted using Lecanemab where the results suggested that the drug may have neuroprotective properties, protecting the neurones against the toxicity of Aβ plaques and fibrils (Söllvander et al., 2018).

A clinical drug trial on humans was conducted by Sturchio et al (2021) based on the theory that the symptoms of AD such as cognitive impairments are linked to a decrease in soluble Aβ rather than increased levels of insoluble Aβ. A drug known as Lacanemab was used in this study, whereby the levels of soluble and insoluble Aβ were compared in 598 patients who showed signs of amyloidosis. Participants were split into three groups; 'normal cognition', 'mild cognitive impairment' and 'AD'. The research had various significant findings; 1) The 'normal cognition' group had higher levels of soluble Aβ than the 'mild impairment' and 'AD' groups, despite the existence of Aβ plaques in the brain. 2) They found that high levels of soluble Aβ were associated with a larger hippocampus (responsible for learning and memory) explaining why the group with the highest volume of soluble Aβ and the presence of Aβ plaques did not show cognitive impairments (normal cognition group). 3) The increased rate of reduction and elimination of insoluble Aβ lead to patients showing moderately less cognitive decline compared to the placebo group after 18 months. In addition to this 4) the patients who also showed decreased levels of soluble Aβ, experienced negative effects as their cognitive impairments worsened. This further supports the idea that high levels of Aβ do not correlate with AD nor cognitive impairment, and that the maintenance of 'healthy' levels of insoluble and soluble Aβ plays a role in cognitive function and the pathogenesis of AD should be the focus for future treatment.

Further research supporting this was conducted, and the results concluded that those who have lower levels of soluble Aβ42 have an increased risk of developing dementia. Those who were deemed more likely to have higher concentrations of Aβ42 plaque in the future but currently had higher levels of soluble Aβ42 exhibited 'normal' brain activity, and their risk of developing dementia over the next 3-years was lower than those with lower levels of soluble Aβ42. This concludes that high levels of soluble

Aβ42 are required for normal brain function, and those with higher concentrations of soluble Aβ42 were at a lower risk of developing dementia (Sturchio et al., 2022).

The majority of the research into the role of Aβ in AD has shown that soluble forms of Aβ are not pathogenic and are required for healthy brain function and cognition, and the main difference between the 'healthy' and pathological ageing and AD brains was the difference in the percentages of insoluble and soluble Aβ, as healthy brains had the highest levels of soluble Aβ and the AD brains had the highest concentration of insoluble Aβ.

People with AD usually live for 8-10 years after being diagnosed, this shows the rate that AD progresses and the impact on a person's cognitive state. As time goes on, the brain deteriorates more and more, thus increasing symptoms of cognitive decline present themselves. AD is a large burden on the NHS as the total cost of care in the UK for dementia patients is £34.7 billion. Each individual with AD in residential care will cost £100,000 a year to care for, meaning the total cost of care for one person with AD based on the typical 8-10 years would be between £800,000- £1 million, or more if they live longer. AD has a major impact on the NHS as well as the country, and the cost of care is predicted to rise over the next 20 years to £94 billion a year (Alzheimer's society, 2018). This alone highlights the importance of further research on AD.

TAU Protein

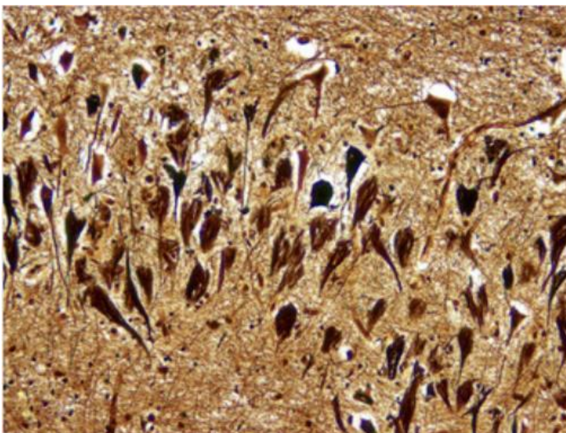


Figure 2. A microscopic image of neurofibrillary tangles caused by the hyperphosphorylation of tau. (Agamanolis, 2020)

Inside the neurone, microtubules congregate to form a cellular highway to allow the transmission of cellular products from the soma to the axon terminal. Tau protein is essential as it plays a role in the pathogenesis of AD, it is a protein that stabilises the structure of microtubules. β-amyloid

activates cellular kinase which phosphorylates tau proteins. If Tau is hyperphosphorylated, it is made to detach from microtubules and begins to aggregate inside the cell to form neurofibrillary tangles (NFT). Microtubules that have been deprived of Tau proteins become destabilised, they collapse which in turn inhibits intracellular transport (DeTure, M.A., Dickson, D.W., 2019). NFTs are formed by many paired helical filaments (PHFs), containing mainly self-aggregated hyperphosphorylated tau (Zhao and Zhao, 2013).

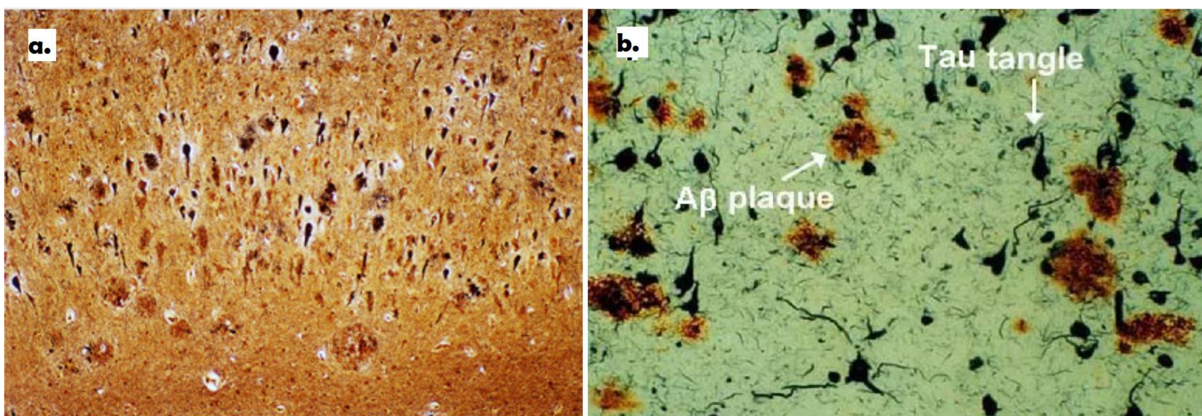


Figure 3. Both A and B are microscopic images of amyloid-β plaque and neurofibrillary tau tangles. Figure A - (Agamanolis, 2020) Figure B- (Manna, 2017)

With extracellular senile plaques blocking the transmission of neurotransmitters at synaptic junctions, and intracellular neurofibrillary tangles inhibiting the transport of molecules, neurones become dysfunctional and can lead to neuronal cell death due to the increase in oxidative stress. This suggests the involvement of oxidative stress in AD-related synaptic loss. Excessive oxidative stress stimulates multiple signalling pathways in the central nervous system which participate in pathophysiological processes leading to cell damage and apoptosis. In AD, the accumulation of Aβ is associated with increased free radicals and increased lipid peroxidation in the brain and an increase in cytokine production (Cunnane et al., 2009). As well as this, evidence has suggested that oxidative stress plays a role in the increased production and aggregation of β-Amyloid, as well as aiding the hyperphosphorylation of Tau, which in turn forms a cycle of increased inflammation promoting the initiation and progression of AD (Zhao and Zhao, 2013).

There is evidence supporting this in experiments involving various AD transgenic mouse models carrying mutants of APP and PS-1, increased hydrogen peroxide levels and nitric oxide production as well as elevated oxidative modifications of proteins and lipids were correlated with the age-associated Aβ accumulation, confirming that Aβ promotes oxidative stress. This also plays a role in some symptoms commonly experienced in individuals with AD.

In the cerebral cortex and hippocampus, the enzyme CHAT (Choline Acetyl Transferase) is produced in Cholinergic neurone soma and travels anterograde to the Axon terminal, where it produces Acetylcholine (ACh) which is essential in learning and the formation of memories.
In AD, there are decreased and dysfunctional cholinergic neurones which show; decreased choline uptake, deceased ACH release, a decrease in CHAT and a decrease in nicotinic receptors.
The reduction of CHAT in AD can be correlated with the number of senile plaques and disease severity.

Pathology of the Disease

Certain structural changes occur in the brain in the presence of Alzheimer's disease, some of these can be seen by macroscopic examination. Most individuals with AD exhibit a reduction in brain weight, and atrophy is found in posterior cortical areas, such as the posterior cingulate gyrus. Atrophy in this area is associated with memory and visuospatial dysfunction. Due to this atrophy, enlargement of the temporal and frontal horns of the lateral ventricle is commonly displayed (figure 4) along with atrophy affecting the amygdala and hippocampus, which is responsible for the formation of new memories. Shrinkage in the hippocampal region is thought to be responsible for short-term memory loss also experienced in Alzheimer's. As well as this, enlargement of the sulcal spaces in the frontal and temporal cortices and the narrowing of the

gyri are usually present, however, the primary motor cortex and the somatosensory cortex are both usually unaffected. The loss of pigmented neurones (Neuromelanin) is found and is represented by figure 1. Moderate cortical damage to areas of the brain such as the structures in the limbic lobe, as well as the association cortices, are commonly found also.

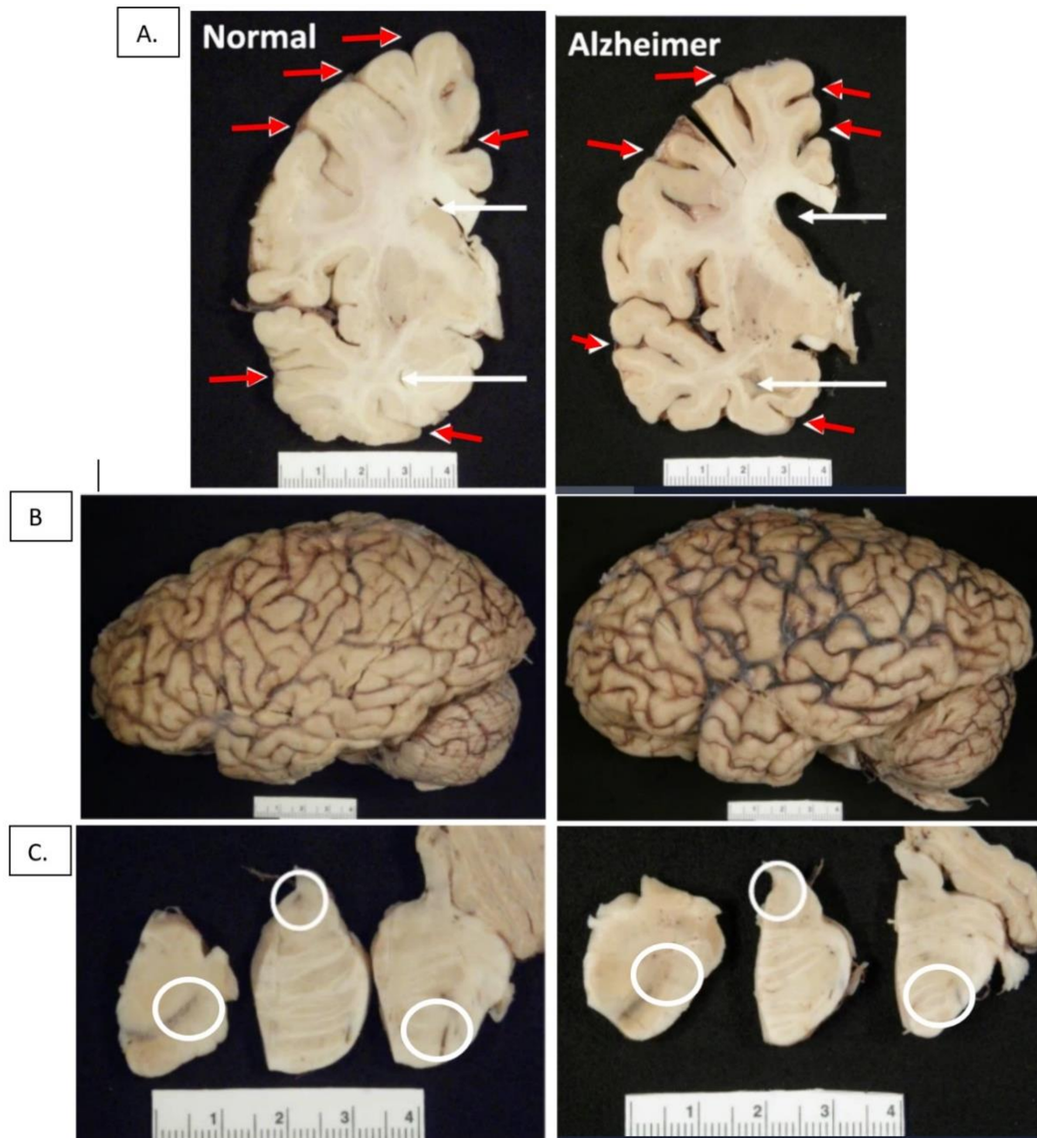


Figure 4. Gross pathology of a brain affected by Alzheimer's disease (DeTure and Dickson, 2019).

The Lateral and coronal sections of the brain in images A and B, it is clear that there is significant widening of the sulcal spaces as well as narrowing of gyri when compared to the healthy brain. This represented by the red arrows in image A, as well as the darkness shown in image B. The white arrows point to areas in which there is enlargement of the frontal and temporal horns of the lateral ventricles. This area is highly enlarged and more prominent in the Alzheimer's brain. Image C shows the loss of neuromelanin in the locus coeruleus, It is found in the healthy brain as it is thought to protect neurones from oxidative stress, however in Alzheimer's brains, some of the neuromelanin has been lost.

It was proposed by Thal et al 2000, that the accumulation of β-amyloid progresses in 5 stages. Stage 1: Isocortical, stage 2: Deposits in the amygdala, hippocampus and entorhinal cortex, stage 3: Accumulation in areas such as the thalamus, hypothalamus, the basal forebrain cholinergic nuclei as well as the subcortical nuclei, striatum and white matter, stage 4: in the substaniata nigra, brain stem structures (medulla oblongata and red nucleus) as well as the superior and inferior colliculi and stage 5: the pons (rape nuclei and locus ceruleus) and the cerebellum. Below, the stages are shown in figure 2, summarized into three stages by Serrano-Pozzo et al, 2011.

Figure 5. PET scan images representing the deposition of Aβ plaque in the brain, shown in three cross sections of the brain (A)- coronal, (B)- axial and (C)-sagittal (Serrano-Pozo, Frosch, Masliah and Hyman, 2011). The accumulation of β-amyloid is shown in three stages in this figure. Stage one, shown in red occurs first, where accumulation is in the isocortex (the six layers of the cortex), followed by stage 2, shown in orange which are the limbic structures such as the amygdala, hippocampus, and the entorhinal cortex. In the stage 3, represented in yellow are the subcortical structures such as the cerebellum, the midbrain, brainstem, and the basal ganglia (Serrano-Pozo, Frosch, Masliah and Hyman, 2011).

Unlike the structural changes found, studies have derived that the density and level of accumulation of Aβ do not correlate with the severity of Alzheimer's, as the build-up of β-amyloid plaque is also found in healthy brains as they age (Figure 3) however increased levels is a hallmark of Alzheimer's disease, along with the structural changes, presence of hyperphosphorylated tau and neurofibrillary tangles.

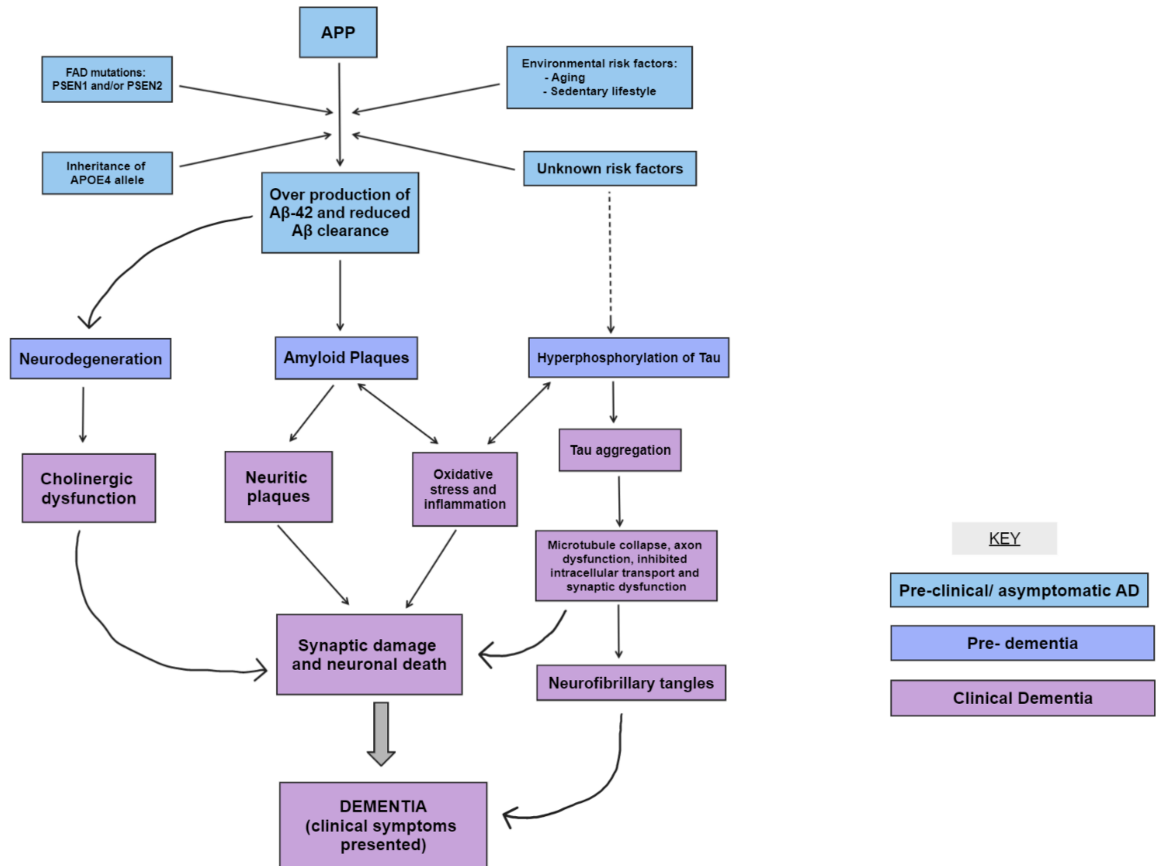


Figure 6. Showing the Pathological cascade of Alzheimer's disease and the mechanisms involved leading to the development of the disease. Dotted arrow representing factors that may contribute to a mechanism taking place. Colour-coded key represents three clinical stages of AD. Light blue representing the pre-clinical and asymptomatic stages of AD where individuals will show no symptoms. Dark blue represents the pre-dementia stage, where an overproduction of Aβ42 is produced, and degeneration is starting to take place. It is thought at this point individuals may show mild cognitive impairment. The purple represents the clinical dementia stage where there will be the presence of neurotic plaques, neurofibrillary tangles and neuronal death taking place. Individuals at this stage thought to show significant cognitive decline (Forlenza, Diniz and Gattaz, 2010).

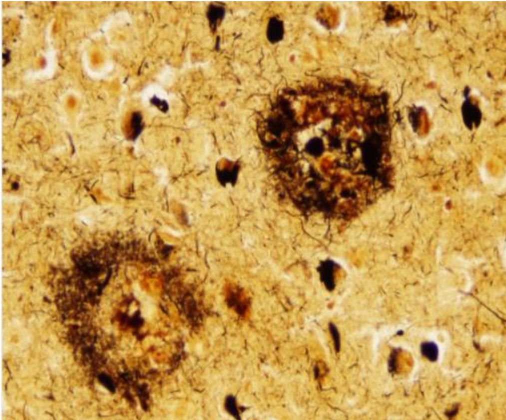


Figure 7. A microscopic image of neurofibrillary tangles and senile plaques as a result of Aβ accumulation after using the Bielschowski silver stain (Agamanolis, 2020)

Aβ is normally broken down in the brain by of process of phagocytosis by microglial cells and by receptor-mediated internalisation by Astrocytes as well as protease neprilysin. In AD, increased levels of Aβ are present due to an increase in production and a decrease in the breakdown of Aβ. Senile plaques block neurotransmitter transmission in the brain and can lead to synaptic death, in conjunction with the added disruption in synaptic transmission due to the presence of neurofibrillary tangles, there is major blockage of communication between neurones as well as high levels of neurotoxicity and oxidative stress (Cunnane et al., 2009).

Genetic Factors

Familial Alzheimer's disease (FAD), also known as early-onset familial Alzheimer's disease (eFAD) is an autosomal-dominant genetic disorder. FAD accounts for up to 6% of all AD cases, the onset ranging from 30-65 years old, compared to 95% accounting for the more common late-set and age-related AD with an onset age of 60-65 years (Bekris et al., 2010). Early-onset FAD is diagnosed in a family when more than one member of the family has AD (usually multiple members affected in one generation) and the age of onset is consistently before the age of 55 years (Bird, 2012).

Genetic mutations linked to the onset of the disease have been identified in three specific genes; Presenilin 1 (PSEN1), Presenilin 2 (PSEN2) and amyloid precursor protein (APP). In contrast, the more common form of AD has one potential risk factor; dysfunctional Apolipoprotein E (ApoE) which is a class of proteins responsible for the metabolism of fat in the body (Bertram and Tanzi, 2004).

There have been over 200 changes in these three genes, some are benign and non-pathogenic however, the role of the other changes has not yet been identified. Further, duplication of APP has also been identified as an additional cause of FAD (Ryan and Rossor, 2010).

Mutations in PSEN1 account for the majority of FAD cases, and this is where most mutations have been found. There have been 177 known mutations in PSEN1, 32 in APP and only 13 in PSEN2 by 2010, and the number has likely increased greatly since (Ryan and Rossor, 2010). Presenilins were initially discovered as the sites of missense mutations of nucleotides, resulting in a change in the coded amino acid which was responsible for FAD. Presenilins are multi-pass transmembrane proteins found to be the catalytic component of γ-secretase and responsible for generating carboxyl-terminal (R-OOH) of the amyloid β-protein from the APP (De Strooper et al, 2012).

There is evidence that PSEN1 has an important role in facilitating proteolytic processes of specific proteins such as the amyloid precursor protein (APP). When PSEN1 mutates, it induces the γ-secretase-mediated processing of APP, thus causing an increase in the levels of Aβ1-42 which increases Aβ accumulation and deposition in the brain, eventually forming the solid Aβ plaques. This was supported by experimental research conducted on the brains of transgenic mice (Thinakaran, 1999).

Research has shown that to date there are more than 30 mutations in the coding sequence for APP, they have been found to either increase or decrease the production of Aβ (Bertram et al.,

2010) however, the majority of these mutations have resulted in an autosomal dominant form of AD with early onset (Proft and Weiss). Most of these mutations have been found to occur near to β- and γ-secretase cleavage sites, increasing Aβ production as they are the enzymes responsible for the processing of APP via the amyloidogenic pathway (Bekris et al., 2010).

Two mutations in the α-secretase (ADAM10) domain were found in members of seven families with known late-onset AD. The two mutations (Q170H and R1q81G) were added to cell lines expressing both APP and ADAM10, the results showed that both mutations resulted in a 70% decrease in the function of ADAM10 (non-amyloidogenic pathway). This resulted in a decrease in levels of sAPPα but increased levels of Aβ production (Kim et al., 2009).

In contrast to this, a study by Jonsson et al. (2012) researched the genome of 1,795 natives in Iceland to identify mutations in the APP coding sequence; It was discovered that this rare new mutation had protective effects against AD and was close to the β-secretase cleavage site. The results showed that this coding mutation known as A673T was more prevalent in the control group (85-year-olds without apparent AD) than the AD group, and resulted in a 50-fold decrease in the production of Aβ. This mutation seemed to decrease the processing of APP by β-secretase, which is known to initiate the production of toxic Aβ via the amyloidogenic pathway. Therefore, finding a way to reduce the use of β-secretase in APP processing could prove beneficial for those who may have mild-moderate AD or are at high risk of developing AD.

A well-known and extensively researched mutation in the β-secretase cleavage site is known as the Swedish mutation. The significance of this mutation is that it results in an increased production of Aβ, resulting in individuals with the mutation developing early-onset AD which is highly heritable (Hsiao et al., 1996). The high rate of Aβ production has made it ideal for use not only in this research project but in many research models of familial AD.

The Swedish Mutation

There have been a few mutations of FAD associated with different regions in the world, such as the Dutch mutation native to the Netherlands, the Arctic mutation and the Swedish mutation. The Swedish mutation is the most widely used to research FAD, and the mutation is used in this research. Native to Sweden, the Swedish mutation is extremely rare and was only found in two Swedish families, this same mutation was not found in the rest of the global population. This familial AD mutation has been found to lead to an increase in the production and secretion of Aβ (Haass et al., 1995) due to mutations at the β-secretase cleavage site of APP (Hsiao et al., 1996), which links it to early-onset Alzheimer's disease. The individuals affected showed signs of memory loss and met the diagnostic criteria of AD a lot younger (>50 years old) than the general public with regular late-onset AD (Shin et al., 2010).

Modelling AD

The Swedish mutation is most generally used on mice models of AD as well as in cell cultures. This mutation significantly enhances the production of APP and thus Aβ. It has been found that mice that have been induced with this mutation (i.e. Tg2576 model) show high levels of Aβ accumulation and then develop AD pathology along with it (Alzforum.org, 2019).

To form a cell line containing the Swedish mutation, the APPswe plasmid is transfected into the commonly used SH-SY5Y neuroblastoma cell line, resulting in the SH-SY5Y APPswe cell line fit for research on FAD (Yin et al., 2012). The SH-SY5Y cells are a human-derived cell line originally established from the bone marrow of a 4-year-old girl with Neuroblastoma in the early 1970s (Hong-rong et al, 2010). These cells have been widely used as a model of neurones since the 1980s as they have been found to possess both the biochemical and functional properties of neurones

(Hong-rong et al, 2010). The SH-SY5Y cells are a subline of the parental cell line SK-N-SH, the original SK-N-SH cells were cloned three times (Kovalevich and Langford, 2013) resulting in the highly versatile SH-SY5Y cells. The SK-N-SH cells contain three phenotypes; Schwannian (S-type), Intermediary (I-type) and Neuronal (N-type). SH-SY5Y cells are the N-type. The neoplastic neural crest-derived cells are excellent for use in various in-vitro studies due to the rate at which they can proliferate, and their ability to proliferate in-vitro is believed to be infinite (Shastry, Basu and Rajadhyaksha, 2001). The proliferation of these cells can occur over a long period of time in-vitro, without becoming contaminated making them great for research (Hong-rong et al, 2010). These cells exhibit properties of stem cells and can be induced to differentiate with the treatment of many agents; the most common being retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) (Hong-rong et al, 2010). When SHSY5Y cells are treated with RA and/or BDNF, the cells differentiate into neuron-like cells. The addition of RA induces the expression of functional TrkB receptors so that the cells are responsive to BDNF. BDNF then activates phosphatidylinositol 3-kinase (PI 3-K) which is responsible for the development of neurites, axons and dendrites as well as cell survival (Jämsä et al., 2004). The SH-SY5Y cells have been used to study a variety of neurological aspects such as; neuronal differentiation, metabolism, neurotoxicity and neuroprotection which play a role in the understanding of neurodegenerative diseases including AD, dementia and Parkinson's disease (Lin-sen et al, 2010).

The cells undergo rapid proliferation and cell differentiation can be induced using different reagents, such as RA; as mentioned above. As well as this, the cells do not have to differentiate to produce APP, as the Swedish mutation cells endogenously produce APP (Klein, 2011) making them a great model for FAD research.

Experimental evidence analysed Aβ metabolism using primary skin fibroblasts from the affected members of the Swedish FAD family as well as their unaffected siblings and spouses. It was found

that the fibroblasts constantly secreted Aβ molecules and there was a 3-fold increase in the release of Aβ found in the skin fibroblast biopsies with the Swedish mutation of FAD.

This was further supported by research showing the results of a Western blot assay conducted on SHSY5Y cells induced with the Swedish mutation, the Swedish mutated cells produced concentrations of APP at a significantly higher level than the control/non-mutated group. The level of Aβ40/Aβ42 was directly linked to the number of mutations present in the cells, thus resulting in the Swedish mutated cells showing the highest ratio of Aβ40/Aβ42 along with the Indiana mutation (Pahrudin Arrozi et al., 2017).

SHSY5Y cells transfected with the Swedish mutation were treated with microRNA (a non-coding RNA which plays an important role in gene expression (O'Brien et al., 2018)) to assess the impact on levels of APP and Aβ40/Aβ42 expression. An ELISA assay was conducted to detect levels of Aβ40 or Aβ42 after treatment, it was concluded that microRNA mimicked β-secretase which inhibited the cleavage of APP via the amyloidogenic pathway (significantly up-regulated in the Swedish mutation of AD), thus resulting in a decrease in levels of Aβ40 and Aβ42 (Li and Wang, 2018).

It can be concluded that the use of SweAPP SHSY5Y cells as a model for AD is effective and has resulted in a lot of research over the years which has contributed to the understanding of AD at a cellular level. The benefit of SHSY5Y cells other than their ability to rapidly proliferate and differentiate into neurones is that when transfected with the Swedish mutation of AD they endogenously produce high levels of Aβ40 and Aβ42 to mimic AD allowing them to be great for research due to their versatility.

Polyunsaturated Fatty Acids

Fats and fatty acids are essential for all living organisms, they make up the cellular membranes, the structural components of cells and tissues as well as regulate biological processes in the body such as brain function (Sokoła-Wysocza, Wysocza, Wagner and Czyz, 2018). The importance of essential fatty acids has been widely researched and is known to play an important role in human health.

Classification

Fatty acids are made up of hydrocarbon chains of variable lengths, containing a methyl group (CH3) at one end and a carboxylic acid group (COOH) at the other. They are classified into two main sub-groups; Saturated and unsaturated. Saturated fatty acids contain all single carbon bonds (C-C) and the maximum number of hydrogen atoms, whereas unsaturated fatty acid chains will contain one or more double carbon bonds (C=C) (Sokoła-Wysocza, Wysocza, Wagner and Czyz, 2018). This paper will concentrate on unsaturated fatty acids which have been found to benefit the human body in many ways, which will be discussed in subsequent paragraphs.

Unsaturated fatty acids can be characterised into 2 groups based on the number of double carbon bonds. Monounsaturated fatty acids contain one double carbon bond, an example of these is Omega-7 and Omega-9, commonly found in berries and olive oil. Polyunsaturated fatty acids (PUFAS) are made up of more than one double carbon bond, examples are the commonly known Omega-3 and Omega-6 (Sokoła-Wysocza, Wysocza, Wagner and Czyz, 2018). This is represented in the figure below.

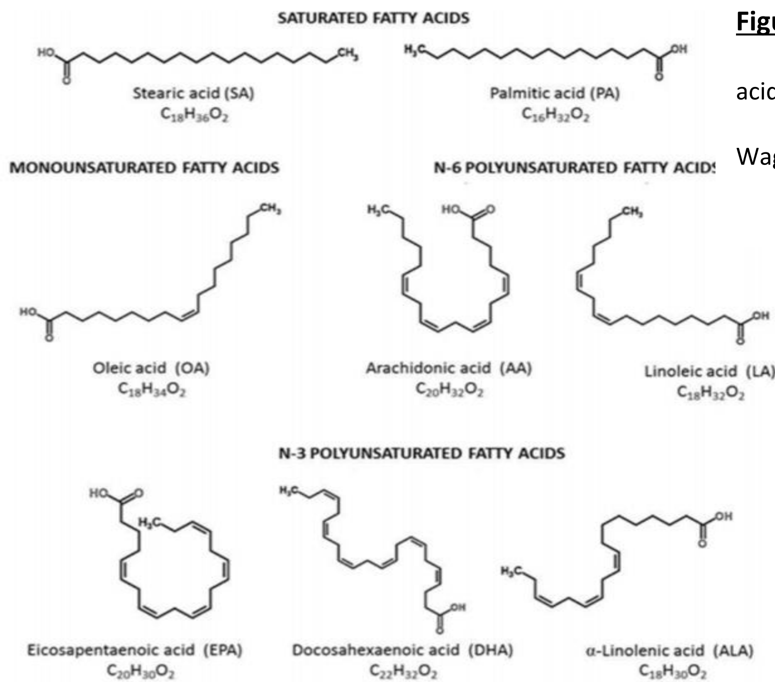


Figure 8. The classification of fatty acids. (Sokoła-Wysocza, Wysocza, Wagner and Czyz, 2018).

PUFAs can be further classified in relation to the location of their first double carbon bond; in Omega-3 PUFAs, the first double carbon bond is located at the third carbon molecule, and it is located at the sixth carbon molecule for Omega-6 (from the methyl end of the chain) (P Ander, MC Dupasquier, A Prociuk and N Pierce, 2003). There are three essential Omega-3; Alpha-linolenic acid (ALA), Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA). ALA is the only Omega-3 that cannot be synthesised by the human body, therefore it must be obtained through dietary sources (Weylandt et al., 2015). ALA is a precursor of Omega-3 and so when ingested, is metabolised into EPA first using enzymes, which can then be further metabolised into DHA. The process of ALA conversion into EPA and then DHA is very limited as it depends on the intake of

ALA and the availability of the enzymes which Omega-6 fatty acids also compete for. Furthermore, this conversion process can only occur in the liver, by astroglial cells and the cerebrovascular lumen, therefore sufficient levels of EPA and DHA are maintained by eating foods rich in Omega-3 (Sokoła-Wysocza, Wysocza, Wagner and Czyz, 2018). ALA is obtained from dietary sources such as seeds; flax, chia and sunflower seeds, as well as nuts and soybean oils (Suzumura et al., 2020). EPA and DHA are found in food sources such as fish (salmon, mackerel, trout) as well as dairy products (P Ander, MC Dupasquier, A Prociuk and N Pierce, 2003).

These Omega-3 fatty acids can also be found in supplement form after research has shown the array of benefits to human health, in particular for cardiovascular diseases after a clinical study reported their ability to prevent the onset of some cardiovascular diseases due to their anti-inflammatory properties. Following this, health agencies around the world allowed the use of PUFAs as a preventative and treatment method for hypertriglyceridemia (the build-up of triglycerides resulting in atherosclerosis) (P Ander, MC Dupasquier, A Prociuk and N Pierce, 2003).

The importance of Omega-3 and DHA

DHA is deemed essential for the health of the nervous system and is required for normal brain function in adults, as well as required to maintain the health of the skin, retina and throughout cellular membranes, especially in neuronal cells and growth cones (Rapoport & Taha, 2014). DHA must be obtained from dietary sources as it cannot be synthesised by the body sufficiently enough to maintain optimal brain health. Therefore, humans and mammals rely on food sources enriched with DHA such as marine animals, dairy products, flax seeds and some nuts to fulfil their required DHA intake. DHA, EPA and omega-3 (overall) also come in capsule form, as a fish-oil supplement (Oria and Pillsbury, 2011).

Benefits of Omega-3

The benefits of ALA, EPA and DHA have been widely researched and found to play a role in several disorders such as; neuropsychiatric, neurodegenerative such as Alzheimer's and Parkinson's disease, as well as, diabetes, cardiovascular diseases and specific types of cancer; prostate and mammary (P Ander, MC Dupasquier, A Prociuk and N Pierce, 2003). These health benefits are due to their signalling properties. DHA, EPA and ALA all have several signalling properties which allow them to have anti-inflammatory effects thus playing a role in the prevention of many diseases (Cipollina, 2015). EPA is a precursor to eicosanoids which are lipid-based signalling molecules (ie. Prostaglandins) these play a role in several bodily processes which contribute to overall health and well being such as immune function and specifically targeted inflammation (Calder, 2013). ALA has been found to have neuroprotective properties, similar to DHA, which play a role in the prevention of neurodegenerative diseases such as AD (Calder, 2012) however DHA plays a strong role in boosting brain health and has been found to have stronger overall signalling properties which reduce brain inflammation (described in further detail in subsequent paragraphs) (Sublette et al., 2011).

Benefits of DHA

Deficiencies in DHA have been associated with chronic diseases such as type 2 diabetes, cardiovascular disease and dementia; Alzheimer's disease (Stark et al., 2016) because of this, the neuroprotective role of DHA is becoming widely researched, and it is being considered as a treatment and preventative option for various diseases. The use of supplements of DHA in mice and humans has shown the presence of an anti-inflammatory response, cell survival and the protection of tissue in microglial cells. DHA could enhance the survival of photoreceptors in the retina, where it inactivated microglial cells so they did not produce an inflammatory response in

mice retina. As well as this, rats with a damaged sciatic nerve were treated with DHA, this significantly reduced neuronal damage as well as neuropathic pain in the mice by reducing the inflammatory cells, such as microglial cells and allograft inflammatory factor 1 (AIF1) and it reduced the expression of pro-apoptotic protein53 in the dorsal root ganglion (Heras-Sandoval, Pedraza-Chaverri and Pérez-Rojas, 2016). Human clinical trials have been used to explore the effect of DHA and EPA on several neurological diseases including epilepsy and stroke. In a trial consisting of 51 epilepsy patients, patients were given 1,700 mg/day of omega-3 supplements, and the frequency of their seizures was reduced in the first six weeks of this treatment, however, this effect was not seen much after the 6 weeks (Yeun et al, 2005). The supplementation of omega-3 did have some benefits, even if they were short-lived. Clinical trials of DHA supplementation in AD patients have displayed mixed and inconsistent results, however, even though several have not shown positive findings there have been some positive clinical trials. DHA has been successful in treating many other diseases and injuries by limiting the inflammatory response and promoting cell proliferation and survival, and there is growing evidence that it plays a positive role in AD also (Cunnane and Chen, 2015).

Epidemiological studies have suggested that increased intake of DHA is associated with a reduced risk of AD. The DHA levels were found to be lower in the brain serum of those with AD, than those without, which can be linked to low dietary intake of DHA or PUFA oxidation (Lim, 2005). Clinical trials on those in the pre-dementia stage of AD showed that when given omega-3 supplements the rate of early memory decline was reduced. This evidence suggests that using omega-3 supplements in the early stages of AD could delay the onset of symptoms, or potentially reduce the risk of AD (Yassine et al., 2017). This means that the optimal time for future DHA/omega-3 trials and treatment can be established with the use of advanced brain imaging techniques.

There have been several in-vitro studies which have researched the impact of Omega-3; specifically DHA, EPA and ALA on SHSY5Y cells induced with Aβ. A study by Hashimoto et al (2011)

used SHSY5Y cells infused with Aβ25-35, following this, cells were then treated with DHA. The results showed that DHA inhibited the neurotoxic effects of Aβ25-35 and improved previous DNA damage caused by Aβ25-35. This highlights the neuroprotective role of DHA against neurodegeneration which occur in diseases such as Alzheimer's and Parkinson's.

Further studies tested the protective role of DHA and EPA using SHSY5Y cells exposed to Aβ, it was found that both DHA and EPA were effective in improving cell viability and reducing oxidative stress in the cells. On top of this, EPA was found to be the most effective out of the two Omega-3s used (Zhang et al., 2018). Further to this, the impact of ALA on SHSY5Y cells induced with Aβ25-35 at concentrations of 1, 2.5, 5 and 25 μg/mL was tested. ALA was found to significantly reduce neuronal cell apoptosis and improve cell viability (Lee et al., 2017). These experiments further emphasise the importance of DHA, ALA and EPA and their ability to reduce inflammation, and oxidative stress and improve cell viability which can play a large role in the potential treatment of neurodegenerative diseases.

DHA Signalling

Two receptors have been highly researched, as the binding of DHA to these receptors has led to treatments for various diseases such as type 2 diabetes and retinal diseases (German et al, 2013). These receptors are; Retinoid X Receptor (RXR) and the G-Protein Coupled Receptor 40 (GPR40), which will be explained in more detail below. The success of this previous research has highlighted the possibility that DHA could be used to treat many more diseases, such as dementia, in particular AD. However, much more research will be required before this can be achieved.

DHA signalling via the RXR receptor

The Retinoid X Receptor (RXR) is a nuclear receptor responsible for cell development, differentiation, metabolism and cell death. RXR is a ligand dependant transcription factor, meaning a ligand must bind to it for it to be activated (Dawson and Xia, 2011). The most common ligand to activate the RXR receptor is 9-cis-retinoic acid (RA), which is naturally stored in the body in the form of retinol (Vitamin A) (Kam et al., 2012). However, PUFAs such as DHA have been identified as RXR ligands (Dawson and Xia, 2011), meaning they can also activate this receptor and its transcriptional functions.

Using DHA as a ligand for this receptor has proved to be successful in delaying the neurodegeneration of target cells. For example, Previous research conducted by German et al (2013) showed that the use of DHA was able to prevent vision loss in neurodegenerative diseases using rat models. DHA was found to promote differentiation of the photoreceptor cells in the retina, as well as delay apoptosis of these cells and promote cell survival by the activation of the RXR pathway. If the activation of the RXR pathway in the retina promotes cell survival and reduces cell death, it shows that it could be possible to delay the degeneration of neuronal cells in neurodegenerative diseases like AD. With this said, it should be possible that these same positive effects can also occur in brain regions affected by AD if these regions are targeted by the RXR- DHA pathway, such as; the cerebral cortex, the hippocampus – the area responsible for learning and memory, the Entorhinal cortex responsible for navigation and perception of time; all which become highly degenerated as AD progresses (National Institute on Aging, 2017). This is an area of research that could prove to be highly successful, and could uniquely treat those with AD.

DHA Signalling via the GPR40 Receptor

The GPR40 receptor is a G-protein coupled receptor belonging to the class of G-protein receptors (Khan and He, 2017). It is one of the seven-transmembrane domain receptors (7TM), this group of receptors mediate cellular responses to various ligands such as odours, pheromones, hormones etc, which make them a good target for the identification of new drugs (Shocichet and Kobilka, 2012; Khan and He, 2017). The GPR40 pathway is activated by PUFAs such as DHA, as this is the natural receptor of PUFAs. When GPR40 is combined with PUFA i.e. DHA, the intracellular signalling pathway is activated, which controls the function of cells (Chen, Gong and He, 2018). GPR40 is ubiquitously expressed in the brain, but it is primarily expressed in the substantia nigra, other areas in which it is highly expressed include the medulla oblongata, hippocampus and the subventricular zone (Briscoe et al, 2002), it can also be found in new-born and mature neurones. Previous research has highlighted that GPR40 signalling in the brain contributes to neurogenesis for memory and learning, which are the primary functions affected considerably but Alzheimer's disease (Nakamoto et al, 2013).

Research that previously tested the GPR40-DHA pathway suggested that it has good therapeutic potential, and an ability to treat diseases such as AD (Khan and He, 2017). In this research, mice were administered Aβ1-42 intracerebroventricularly to impair their cognitive abilities similar to how it would affect those with AD. After this, they were treated with a GPR40 receptor agonist (GW9508) to trigger the GPR40 pathway to uncover what effect this would have. The mice in the treatment group experienced enhanced ability in learning and memory when performing spatial learning and memory tasks such as the Morris water maze test. The results suggested activation of the GPR40 receptor induced an increase in brain-derived neurotrophic factor (BDNF), nerve growth factor and Neurotrophins 3 and 4. It can be concluded from the results that, not only does GPR40 contribute to neurogenesis, but it also possesses neuroprotective functions which would promote

cell survival and reduce cell death, meaning it could be used to treat and slow down the progression of AD (Khan and He, 2017).

Now there is evidence that the GPR40 pathway when activated by an agonist plays a role in neurogenesis and neuroprotection, and it is known that DHA has neuroprotective properties itself. Therefore, when GPR40 is combined with DHA and the pathway is activated there is great potential that this could have highly neuroprotective effects on the cells being damaged in AD, and so it could slow down the rate of the degeneration of these neurones, which hypothetically will be of great benefit to those with AD, as their rate of cognitive decline could slow down or even stop.

However, only a few studies have examined the role of GPR40 under physiological conditions (Nakamoto et al, 2013). With this said further research into the GPR40-DHA pathway needs to be carried out to determine whether targeting this pathway could benefit those with AD in terms of improving learning, memory and cognitive abilities.

Current Treatments for AD

Currently, there is no known cure for AD, however, drug and non-drug treatments paired together have been found to alleviate both the cognitive and behavioural symptoms and have helped many individuals with AD to manage their symptoms. However, all the drug treatments available to the general public are symptomatic; meaning they only treat the symptoms of the disease, making it more manageable for people to live with AD. Due to the increasing life expectancy, the prevalence of AD has vastly increased and the number of people with AD is increasing, This has led to extensive research into possible treatments for AD which target the cause of the disease rather than the symptoms. If these were discovered, they would increase the quality of life for people with the disease and their family members. Drugs targeting the pathological molecule of the disease; the Aβ

plaques are what scientists have been aiming to achieve for many years, yet there are no effective therapeutic options to prevent or treat AD (Yiannopoulou and Papageorgiou, 2012).

Treatment of Neurotransmitter Disturbances

The current well-established drug treatments available target the neurotransmitter disturbance aspect of the disease which is associated with common symptoms such as depression and anhedonia such as the commonly used cholinesterase inhibitors (CI) used to treat all stages of AD. It is believed that the Cholinergic system in the basal forebrain is affected in the early stages of the disease, this leads to the loss of acetylcholine (Ach) neurones, which are responsible for processing memory and earning. Due to the loss of Ach neurones and their enzymes, Ach cannot be synthesised or degraded which ultimately leads to memory loss as well as the deterioration of other cognitive and psychiatric functions (Yiannopoulou and Papageorgiou, 2012).

Psychological symptoms are common in all stages of AD, even in the mildest form, it increases in severity as the disease progresses. These are the main factors that cause patients to be institutionalized as their caregivers, usually, family members cannot deal with the burden (Yiannopoulou and Papageorgiou, 2012). An observational study by Zec and Burket in 2008 determined that the behavioural and psychological symptoms of AD can be characterised into four main categories based on the symptoms shown. These are; Hyperactivity - 64% (e.g. aggression and disinhibition), Apathy - 65% (e.g. lack of interest and enthusiasm), affective symptoms – 59% (affecting mood i.e. depression and anxiety) and lastly, psychosis – 38% (e.g. hallucination and delusions). It is believed that the conventi9oanl AD drugs maybe reduce the behavioural symptoms mentioned above, however when these symptoms become extreme, the AD drugs may not be as effective in treating these aspects as that is not their primary target, therefore other drugs may be

given alongside such as antidepressants and antipsychotics, which have been found to alleviate the behavioural and psychological aspects of the disease (Ballard and Corbett, 2010)

Current drug treatments for AD have weak beneficial effects on cognitive function as well as on behavioural and psychological symptoms. The discovery of new drugs that act during the early stages of AD could be considered a 'medical need' and early intervention is critical because is it thought that a delay in treatment is associated with increased disease progression, and non-reversible symptoms (Mancuso *et al*. 2011).

Treatments targeting Amyloid-β in AD

For the most effective preventative treatment, action should be taken well before the symptoms of AD occur and are diagnosed, this is because research has shown that some AD biomarkers such as Aβ accumulate in the brain around 20 years before the onset of the disease and before the common symptoms show (Rygiel, 2016). This would be extremely difficult for the general population as they are not at as high risk of developing AD at a younger age compared to the population of those with FAD. Moreover, those with FAD or who are at high genetic risk are usually well aware of their susceptibly to the disease, so for them, preventative measures would be an obvious precaution to take in the future when this type of treatment has been fully established. But this leaves a gap in the treatment area, as research should be conducted to discover a safe and effective disease-modifying drug (Rygiel, 2016) that works to prevent the development of AD in those with a genetic predisposition as well as the general public by targeting the neurotoxic Aβ before the damage is done.

There has been great research into this approach over the last 10 years, and there have been many potential drugs tested to work against Aβ. These anti-amyloid methods are responsible for reducing

the production (via γ and β secretase inhibitors) or amplifying the clearance (via monoclonal antibodies; described further in text) of Aβ. Monoclonal antibodies are made by identical immune cells which are clones of the unique parent cell, and so they will bind to the same epitope (Prins and Scheltens, 2013) Monoclonal antibodies vary based on their immunoglobin G class, some of these can be used to bind to the senile plaques and some bind to the soluble Aβ (Rygiel, 2016). These novel methods have been tested for the past decade, hoping they can clear and degrade the senile and Aβ plaques. This method of treatment known as immunotherapy is the most innovative of treatments for AD right now which is characterised by two subtypes of immunisation (Rygiel, 2016); these are passive immunisation and active immunisation. The passive method involves the use of humanised monoclonal antibodies which are known as anti-Aβ antibodies, predicted to clear the Aβ species either directly or indirectly via microglial cells. They are administered either by intravenous infusion or subcutaneous injections. This treatment is thought to cease neurodegeneration by stopping the amyloid cascade, and 2 large-scale clinical trials have been conducted on people with AD that reached phase 3, however, had to be terminated after failing. The drugs involved are known as Solanezumab and Bapinezumab (Prins and Scheltens, 2013). Currently, there are ongoing trials in people who are genetically predisposed to developing AD and those who show biomarkers associated with the presence of Aβ in the brain already (Rygiel, 2016). However, more clinal trials are to be conducted using monoclonal antibodies as they have great potential to treat AD, there were some beneficial cognitive effects seen in the last clinical trials, despite them being terminated, but much more research is required as they are in the early stages of developing such treatments for AD. Hopefully, in the future, there will be more advanced methods to effectively treat AD, and prevent it from progressing in those living with it. This would greatly improve the quality of life for individuals with AD and their family/carers and people around them.

Treatments targeting the RXR Pathway

It is believed that an RXR agonist could potentially be a viable treatment method because the binding of the agonist to the RXR would activate the receptor's transcriptional functions and would induce apoE expression. This would facilitate Aβ clearance, induce the expression of apoE and assist microglial phagocytosis (Cramer et al., 2012). Thus, Aβ accumulation will lessen, as it will be cleared and degraded before it can accumulate to form Aβ plaques, which are the main hallmark of AD and are associated with loss of neuronal function in the brain. Bexarotene, originally an anticancer drug was researched as a possible treatment for AD as it is an RXR agonist, and it can penetrate the blood-brain barrier. It has been found that Bexarotene can improve cognition and memory in mice models of AD. As well as it showing to be useful in mice models of ALS, stroke, Parkinson's and Multiple sclerosis (Mariani et al., 2017). Bexarotene was tested on APPswe/PS1 mice models, the treatment assisted the clearance of soluble Aβ1-42 and rapidly lowered the levels of Aβ1-40 and Aβ1-42 in the brain interstitial fluid (ISF) 6 hours after administration, and levels reduced by 25% at 24 hours. It was found that the half-life of Aβ in ISF was reduced from 1.4 hours to just 0.7 hours, meaning it could be clear at a much faster rate, and that's why the levels of Aβ in ISF were decreased (Cramer et al, 2012). This is represented in the figure below.

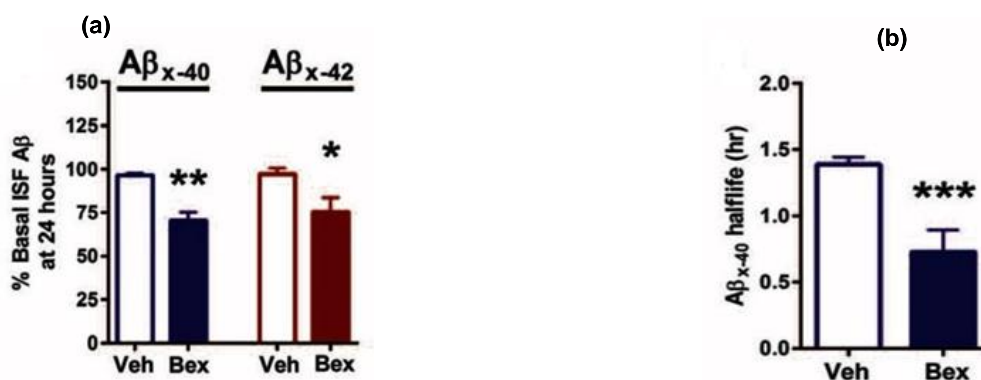


Figure 9. (Cramer et al, 2012) **Figure a.** Showing the effect of Bexarotene on ISF Aβ levels after 24 hours compared to the control (Vehicle). **Figure b.**The reduction in half-life after Bexarotene administration compared to the control (Vehicle).

Another piece of research supporting the benefits of Bexarotene was conducted on five mice models of FAD. These transgenic mice display APP deposits and Aβ peptides within their neurones in layer V, and these accumulations appear in the mice in the early stages of the disease, before any extracellular plaques form. The mice model showed neuronal death in pyramidal neurones as well as in layer V of the cortex, neurite dystrophy and they showed behavioural impairments before Bexarotene (Mariani et al., 2017). The mice were administered Bexarotene for 15 days, and results showed that the drug promoted neuronal survival by 44%, which is evidence that Bexarotene has neuroprotective effects and can help reduce neuronal apoptosis (Mariani et al 2017). However, following 15 days of bexarotene treatment, the same mice FAD mice were analysed for the presence of plague using 6E10 staining. The stain analysis showed a great reduction in soluble and insoluble Aβ1-42 in the cortex after 15 days of treatment due to bexarotene's ability to increase the expression of APOE4. The same reducing effect was not found for the more aggressive Aβ1-42 (Mariani et al, 2017).

It is clear that Bexarotene improved the AD pathology in the transgenic mice models in terms of reducing the accumulation of Aβ plaques and promoting neuronal survival, showing it has great potential to treat AD in the future; clinical trials are ongoing testing the use of Bexarotene in humans, and hopefully, they will be successful, as novel treatments are required.

DHA Treatment

DHA is known to have neuroprotective effects on the brain, which have been thought to enhance cognitive ability in those who regularly consume diets enriched with omega-3 fatty acids or fish, thus reducing cognitive decline and lowering the risk of AD development. Those with AD had lower levels of DHA in the brain, whether this is a cause of AD or an effect of the disease is unknown. But what is known is that the brain's ability to produce DHA itself is limited, and therefore to increase levels of DHA in the brain, it must be sourced from the diet (Pan, Khalil and Nicolazzo, 2015).

Evidence Supporting the Use of DHA as a Treatment Method

Many epidemiological studies have highlighted that increased seafood and fish consumption can protect the elderly from developing cognitive impairments and dementia; in particular, AD Evidence from two correctional studies showed that PUFA intake from marine sources did not lower the risk of cognitive impairments in old aged adults, but it did lower the risk in those that were middle-aged (Kalmijn et al, 2004; Le Coutre and Montmayeur, 2010). Moreover, a study monitoring the elderly showing cognitive impairments for 3-5 years concluded that consuming 105g or more of fish per week lowered the rate of cognitive decline from 75% to 10%. However, the issue with this was that the effect of DHA/ omega-3 intake did not always present the volume of fish intake, as different species of fish will contain different levels of omega-3 PUFA, and some will contain higher levels than others (Le Coutre and Montmayeur, 2010). Therefore, the amount of fish consumed in weight should not be relied on to represent the level of omega-3 PUFA and therefore decreased risk of dementia; particularly AD.

Evidence Against DHA as a Treatment Method

However, previous research linking omega-3 (DHA) and cognitive decline was insignificant (Gillette Guyonnet et al., 2007; Plourde et al., 2007; Le Coutre and Montmayeur, 2010). There are many benefits of consuming increased levels of Omega-3/ DHA, however, consuming large amounts of fish daily may not be great as there are potential side effects that can occur from consuming more than 1 g/day, studies have suggested. It is low risk to consume up to 1g/day of omega-3, but intakes that are higher than this can increase blood glucose levels, the concentration of cholesterol and low-density lipoprotein (LDL) as well as increasing the risk of gastrointestinal irritation (Kris-Etherton et al., 2002; Erdman, Oria and Pillsbury, 2011). As well as this, people must consider

environmental contaminants such as mercury which can pose a potential risk as it can accumulate in some species of fish, this risk can be decreased by not consuming specific fish such as mackerel and swordfish. Excessive intake of omega-3 PUFA can prevent the synthesis of eicosanoid thromboxane, which is responsible for platelet aggregation. Therefore, increased intake can lead to an increased risk of bleeding. However, it does seem rare, as it has not been observed in several clinical trials using fish oil supplements (Huang et al., 2007; Javierre et al., 2006; Oria and Pillsbury, 2011). With that said, Fish oil/ Omega-3 supplements are widely available in supermarkets and are generally good to include in your diet if you do not receive adequate levels of omega-3 from your normal diet.

Because the results of clinical trials and observational studies have been mixed when methods such as omega-3/DHA supplements and increased fish intake have been used. This suggests that new methods of administering DHA should be established, as there is potential for DHA and omega-3 to protect the brain from inflammation and neuronal loss found in AD, as it has been highlighted in some studies, and it is known that DHA has neuroprotective and anti-inflammatory effects; further research is required to find novel methods that could prove more successful, and provide consistently positive results such as intravenous administration of DHA, which could have more immediate effects on the brain.

This experiment has been influenced by previous studies, but it is not a direct replication of one particular study. Previous research which influenced this experiment includes Arrozi et al (2017) Zhang et al. (2018), and Dyall et al 2015 who have all researched the neuroprotective effects of Omega-3 in models of AD. The uniquity of this experiment is that it encompasses the use of Swedish mutation and Native SHSY5Y cells to model AD, and analysing the potential therapeutic role of DHA, EPA and ALA on these models.

AIMS

Main thesis aim: To examine the neuroprotective role of PUFAs on Swedish SHSY5Y cells

Aim 1: Is there a difference in cell viability between Native and Swedish SHSY5Y cells?

Aim 2: Is protection against cell death in Swedish cells specific to DHA treatment?

Aim 3: Does PUFA treatment affect the volume of B-amyloid produced in Swedish cells?

Hypothesis: DHA will be the most effective in improving the cell viability of Swedish SHSY5Y cells and reducing levels of Aβ42

Methods

Cell Culture

The SH-SY5Y (Swedish mutation and native) cell line was acquired from Liverpool University where some of the SHSY5Y cells were transfected with the SweAPP mutation to generate the SHSY5Y-swe cells. The cells were cultured in T75 flasks containing 20ml Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) with the addition of 10% Foetal Bovine Serum, 1% Non-essential amino acids, 100nM GlutaMAX (1%), 10µg/ml Streptomycin and 100 units/ml Penicillin. Cell cultures were incubated at 37^{0}c in 5% CO2 until the flask became 70% confluent. The medium was changed every 48 hours.

Cell Plating

The cells were lifted from the T75 flasks when 70% confluency was reached using 2 ml of 0.25% trypsin (a proteolytic enzyme). After 2-3 minutes, the cells were examined under a microscope to check that they had been lifted from the base of the flask. When 90% or more of the cells had

lifted, the trypsin was inactivated by adding a further 10ml of medium to prevent damage to the cells. The cell suspension was then transferred to a tube and centrifuged at 1000 rpm (repeats per minute) for three minutes. The supernatant was carefully removed, leaving behind the cell pellet which was gently re-suspended into medium solution. A 10ul sample of the cell solution was taken to determine cell density and cell viability before plating for experimentation. This was confirmed using trypan blue staining and cell counts (explained in detail below). The cells were plated at a density of 2.0×10^{5}, which is roughly calculated to be between 120 and 140 cells. 10μl of cell/medium mix was pipetted into each well of a 96-well, a 12-well or a 6-well plate. The cells were differentiated into neurones once plated, using a differentiation media containing the maintenance medium and 10μM retinoic acid (RA). These cells were treated with RA for three days, to allow for cell differentiation before the next stages of the experiment continued.

Trypan Blue Cell Counts and Time-Based Survival

The cells were lifted using trypsin, centrifuged and re-suspended (as described above) in phosphate-buffered saline (PBS). An aliquot of the cell suspension was mixed with an equal volume of 0.4% trypan blue solution. Using a Neubauer chamber, the cells were counted under a light microscope. Dead cells were positively stained with trypan blue, and living cells remained white/clear. The cells stained with trypan blue were used to express the percentage of cell death in the total cell population of stained and unstained cells, from this cell viability was calculated and compared between SH-SY5Yswe cells and native SH-SY5Y cells. Trypan blue was conducted on Swedish and Native cells at time points; 24 hours, 46 hours, 72 hours and 96 hours which allowed cell viability to be measured over time. It was used in the MTT assay and Fatty Acid treatment experiment.

MTT Assay

MTT assay was used to determine cell viability by measuring cell metabolic activity, MTT assay was carried out following PUFA treatment to the cells. This assay is based on the reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide). Metabolically active cells reduce MTT with the use of NAD(P)H-dependent oxidoreductase enzymes, which converts MTT into purple formazan crystals. The formazan crystals can be dissolved using DMSO (Dimethyl sulfoxide), the absorbance of this solution is used to estimate the percentage of viable cells. Typically, the darker the solution, the greater the number of viable and metabolically active cells.

MTT salt was dissolved in the culture medium at one milligram of MTT per one millilitre of medium. The medium was removed from the 96-well plates containing experimental cells and replaced with 50 µl of MTT solution per well and incubated at 37ºC for 2 hours. The MTT solution was then removed and 100 µl of DMSO was added to each well to dissolve the formazan crystals. A Microplate Reader was used to measure the absorbance of the solution at 560 nm.

Aβ ELISA Assay

ELISA is an enzyme-linked immunosorbent assay used to detect and quantify proteins and antigens in samples. The level of Aβ 1-40 and Aβ 1-42 were detected following the ELISA protocol. The tubes containing the cell samples were weighed to determine the weight of the sample. The levels of Aβ 1-40 and Aβ 1-42 present in the tissue were ascertained by following the protocol for the ELISA (enzyme-linked immunosorbent assay) method using E_{max} Immunoassay System (Promega, Madison, Wisconsin). The tissue was homogenized and centrifuged, and 100 µL of

supernatant fluid containing the extracted Aβ was aliquoted in duplicate into 96-well plates precoated with anti- Aβ 1-40 or Aβ 1-42 monoclonal antibody. Following blocking to reduce nonspecific reactions, the captured Aβ 1-40 or Aβ 1-42 was incubated with 1:500 antihuman Aβ 1-40 or Aβ 1-42 polyclonal antibody (Promega). The specifically bound polyclonal antibody was detected using anti-IgY conjugated with horseradish peroxidase antibody, which when incubated with a chromogenic substrate resulted in a colour change proportional to the amount of Aβ 1-40 or Aβ 1-42 present. The 96-well plate also included duplicates of serially diluted BDNF (supplied in the ELISA kit) samples of known concentration ranging from no Aβ 1-40 or Aβ 1-42 to 500 pg/mL, with 6 data points in between. The absorbance of the colour development was measured in a microplate reader at a wavelength of 450 nm to allow quantification of the Aβ 1-40 and Aβ 1-42 concentrations present in the test samples based on the absorbance from the standardized samples of known concentrations. The total protein concentration in each sample was determined using a Bradford protein concentration assay using known concentrations of bovine serum albumin to generate a standard curve. Concentrations of Aβ 1-40 and Aβ 1-42 were expressed as a ratio of Aβ 1-40 and Aβ 1-42 against total protein concentration. The concentrations in each sample were determined in duplicate, and the mean value was used for analysis.

Fatty Acid Treatment

Swedish and Native cells were plated separately into 96-well plates with medium, 100µl of cell/medium solution was added per well and it was plated in triplets. Four plates were used, one plate per time-point (24, 48, 72 and 96 hours after PUFA treatment). Three fatty acids were to be used: DHA, ALA, and EPA. Various volumes of fatty acid were added to the medium to make up three concentrations: 0.1µM, 1µM and 10µM. Cells were then treated with 100µl of fatty acid, at the three concentrations as well as a vehicle (1µM ethanol) and a control (medium alone). The

cells were then incubated at 37^{0}c in 5% CO_2. At each time point, a plate was taken out for further experimentation. The treated cells were then used in the MTT assay and Trypan blue cell counts to calculate cell viability at each time point.

Statistical Analysis

The data produced by the experiments were analysed using Prism-Graph Pad statistic software and it is believed that ANOVA tests were used. Once the analyses were completed the graphs were produced on Prism before being exported. The data are presented as mean values and the error bars represent standard error (SE). Where the standard error bars have not been added due to the group (native or control) being set to 100% and all other values being measured relatively. The F-values represent the differences between the two cell lines (Native and Swedish). N-values represent technical repeats, as the same cell sample was used and repeated 3 or 5 times depending on the n-value shown in the graphs, also displayed by the raw data found in appendices 1-5.

Results

Is there a difference in cell viability between the two cell lines (Swedish and Native)?

SH-SY5Y neuroblastoma cells were treated with RA, to induce cell differentiation allowing the cells to have a neurone-like phenotype. The viability of cells was compared against the native and Swedish cells. In the Swedish cell group, cell viability decreased over the 96-hour time period, showing a significant difference in cell viability between Swedish cells and native cells (F (3, 32) = 3.215, P=0.0358). At 24 hours, the cell viability of Swedish cells was 91%, compared to 100% in the native cell group; which remained at 100% throughout all time points. The native cell group was the control.

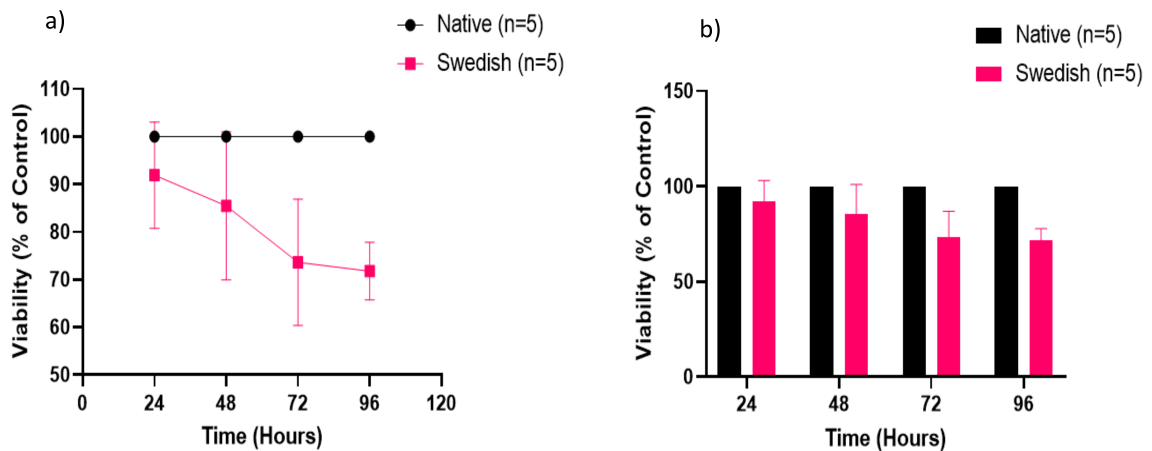


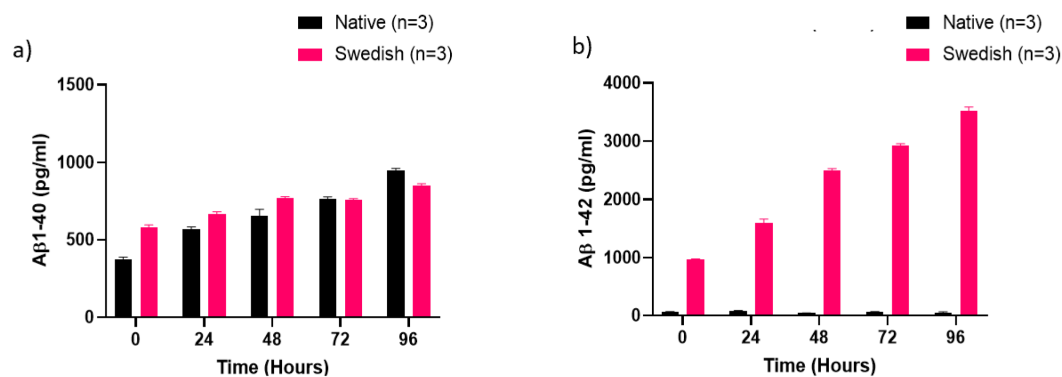
Figure 10. **MTT analysis of cell viability of SH-SY5Y Native and Swedish cells. Cell viability measured at 24, 48, 72 and 96 hours.** A line-graph representing cell viability of both cell groups from 0 to 96 hours (A). A bar-chart representing cell viability of both cell groups from 0 to 96 hours (B). Standard error bars added to Swedish cell group. No standard error bars on native cell group as viability was set to 100%, and Swe cells were compared in relation to native cells.

Between 48 and 72 hours, there was a large decrease in cell viability in the Swedish cell group, from 85% to 74%. Followed by a further decrease to the lowest value of 71% by 96 hours. This

shows a rapid decrease in cell survival in Swedish cells, which was significantly lower compared to the Native cells which remained at 100% viability after 96 hours (F (1, 4) = 38.55 P= 0.0034).

Is there a difference in the volume of Aβ1-40 and Aβ1-42 produced between the two cell groups (Swedish and Native)?

Native and Swedish cells underwent an ELISA assay to quantify the levels of Aβ 1-40 and Aβ 1-42 being produced. The volume of Aβ was measured over 96 hours. Figure (a) shows that over 96 hours the level of Aβ 1-40 gradually increases, with no significant difference between native and Swedish cells (F (1, 2) = 173.0, P=>0.005). However, the level of Aβ 1-42 significantly increases over time (Fig 10b: F (4, 20) = 1319, P<0.0001) in the Swedish cell group, with a significant difference between the native and Swedish cells (F (4, 20) = 1339, P<0.0001).



<u>Figure 11.</u> Elisa assay on SH-SY5Y Native and Swedish cells quantifying production of Aβ 1-40 and Aβ 1-42 at 0,24,48,72 and 96 hours. A bar chart representing the volume of Aβ1-40 produced between 0 to 96 hours (A) for both cell groups. A bar chart representing the volume of Aβ1-42 produced from 0 to 96 hours (B) for both cell groups. Standard error bars applied to both native and Swedish cell groups.

In the native group (figure b) Aβ1-42 ≈ 100 pg/ml at 0 hours, and stays constant throughout, compared to Aβ1-42 at 1000 pg/ml in the Swedish cell group at 0 hours. At 24 hours Aβ1-42 ≈ 1800 pg/ml, which is significantly different to the level of Aβ1-42 at 24 hours in the native cell group remaining at ≈ 100 pg/ml. At 48 hours Aβ1-42 ≈ 2500 p/ml, at 72 hours Aβ1-42 ≈ 3000

pg/ml and 4000 pg/ml at 96 hours. Results displayed that the Swedish cells produced a significantly higher volume of Aβ 1-42 compared to native cells (F (1, 2) = 9867, P=0.0001).

MTT analysis: Does PUFA treatment (DHA, ALA and EPA) affect cell viability across both SH-SY5Y cell groups (Native and Swedish) following 24hr, 48hr, 72hr and 96hr treatment?

Native and Swedish cells were treated with three concentrations of DHA, EPA and ALA (0.1µM, 1µM and 10µM) and cell viability was measured over 24, 48, 72 and 96 hours. The MTT analysis shows that at every time point, cell viability was significantly higher in the Swedish cell group treated with DHA 1µM (F (10, 40) = 6.416, P= <0.0001) and DHA 10µM (F (10, 40) = 6.416, P= <0.0001) compared to all other fatty acid treatment groups at all other time points. There was no significant effect of PUFA treatment on the native cells at any concentration or time point (F (10, 40) = 6.416, P= >0.005).

At 24 hours (Figure a) cell viability was significantly higher when Swedish cells were treated with

1µM (F (1, 4) = 3.976, P= <0.0001) and 10µM of DHA (F (1, 4) = 3.976, P= <0.0001).

With 1µM DHA treatment, cell viability reached ≈ 140%, and ≈ 136% with 10µM DHA, both significantly higher than cells treated with 0.1µM DHA (F (1, 4) = 3.976, P= <0.0001). Swedish cells treated with 1µM DHA and 10µM DHA showed a significant increase in cell viability compared to cells treated with EPA or ALA (F (1, 4) = 3.976, P= <0.0001). 1µM ALA had the lowest percentage of cell viability at ≈106%, however, it was not significantly less than EPA treatment (F (1, 4) = 3.976, P=0.1169). There was no significant difference in cell viability between EPA and ALA for Swedish cells (F (1, 4) = 3.976, P=0.1169). There was also no significant effect of any PUFA treatment on the native cells at 24 hours (F (1, 4) = 3.976, P=0.1169).

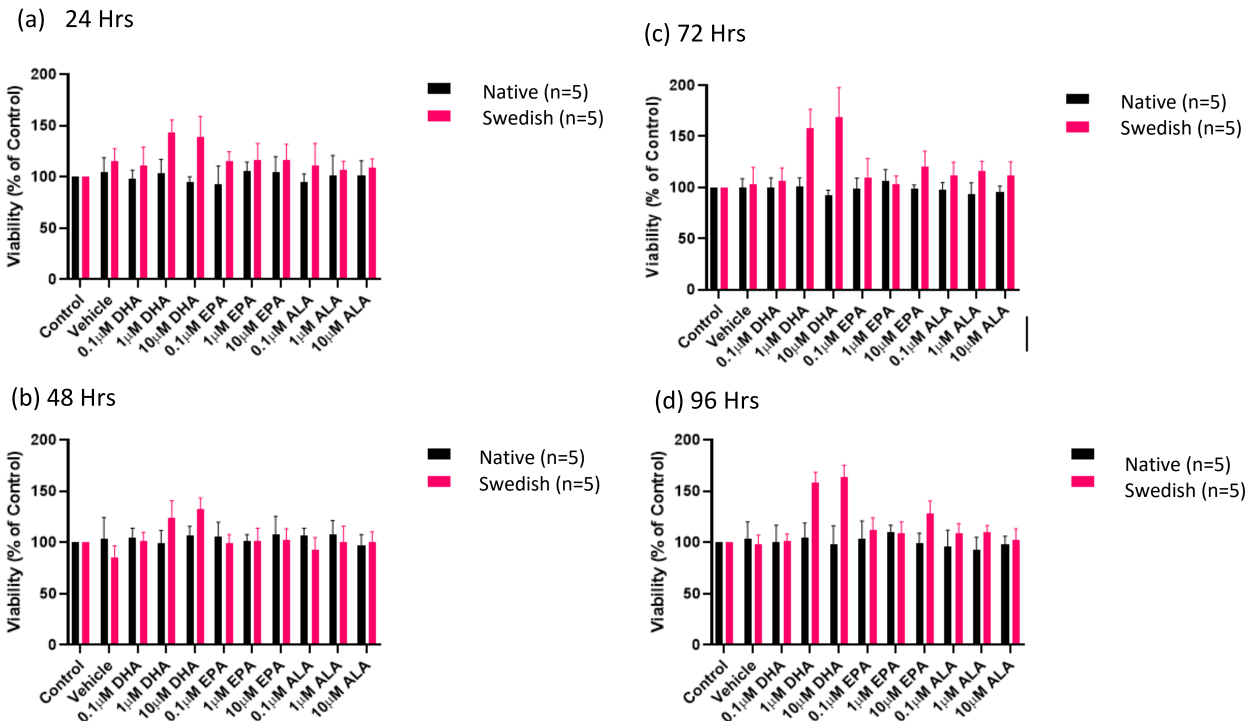


Figure 12. **MTT analysis of cell viability of SH-SY5Y cells (Native and Swedish) following PUFA treatment.** Four bar charts produced from cells treated with DHA, EPA and ALA at concentrations of (0.1µM, 1µM and 10µM) at 24 hours (A), at 48 hours (B), at 72 hours (C) and at 96 hours (D). Standard error bars added to both native and Swedish cell groups. No standard error bars on control groups as they were set to 100% viability and treatment groups were relative to control group.

At 48 hours (Figure b) in the Swedish cell group, the viability of cells treated with 1µM DHA was significantly higher than cells treated with any concentrations of EPA and ALA (F (10, 88) = 3.373, P=0.0009). Also, cells treated with 10µM DHA had a cell viability of roughly 132%. There was no significant difference between Swedish cells treated with 1µM DHA or 10µM DHA F (1, 88) = 0.006154, P=>0.005). However, cells treated with either 1µM DHA or 10µM DHA had significantly higher cell viability than all other PUFA treatment groups, including 0.1µM DHA (F (10, 88) = 3.373, P=0.0009). There was no significant difference in cell viability between native or Swedish cells treated with 0.1µM DHA (F (1, 88) = 0.006154, P=>0.005). In addition, in the native cell group, there was no significant difference in cell viability across all PUFAs (DHA, EPA and ALA) and their various concentrations (F (10, 88) = 3.373, P=>0.005).

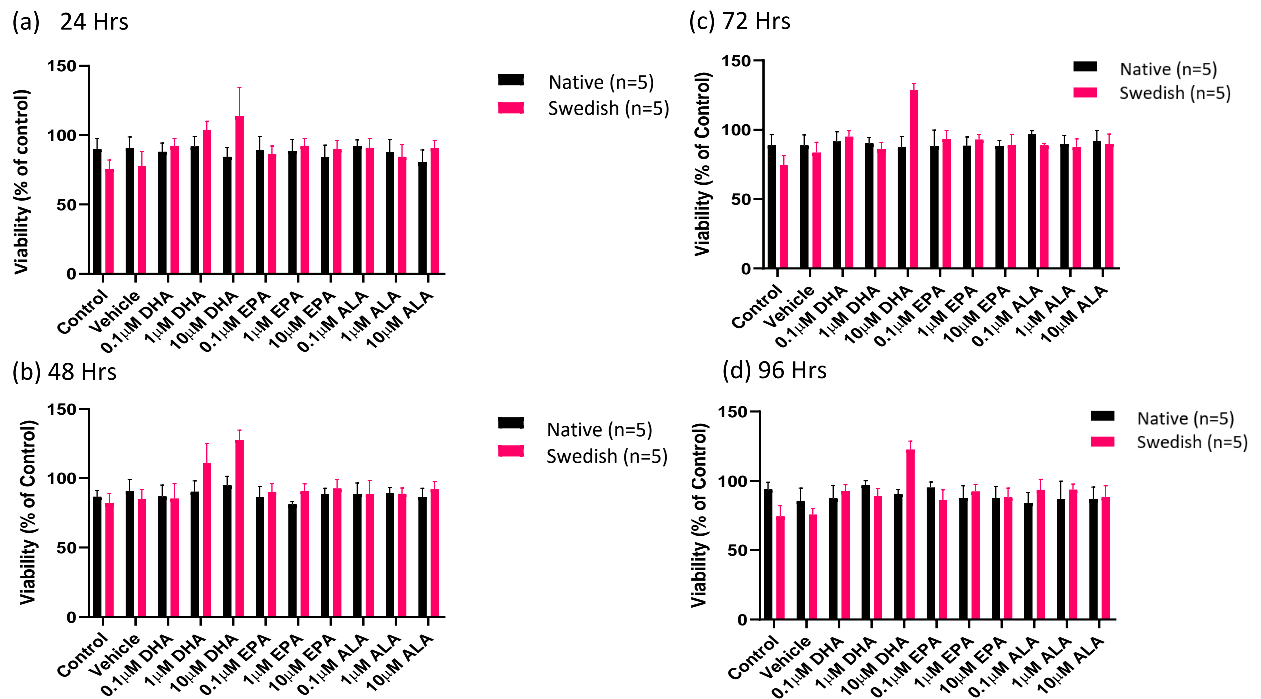
The most significant increase in cell viability is displayed at 72 hours post-1µM DHA ($F (10, 88) = 9.861$, $P<0.0001$) and 10µM DHA treatment in the Swedish cell group (Figure c). There was a significant increase in cell viability in cells treated with 1µM DHA and 10µM DHA compared to all other treatment groups ($F (10, 88) = 9.861$, $P<0.0001$). There was no significant difference in cell viability between 1µM DHA and 10µM ($F (10, 88) = 9.861$, $P=>0.005$). However, there was a significant difference in cell viability between 0.1µM DHA and 1µM DHA, as well as 0.1µM DHA and 10µM DHA ($F (10, 88) = 9.861$, $P<0.0001$). Swedish cells treated with EPA, ALA and 0.1µM DHA displayed similar cell viability, and there was no significant difference between these values ($F (10, 88) = 9.861$, $P=>0.005$). However, at 0.1 µM EPA, there was a significant increase in cell viability in the Swedish cell group compared to the native cell group ($F (10, 88) = 9.861$, $P<0.0001$). There was no significant difference found in cell viability between Swedish and native cell groups ($F (10, 88) = 9.861$, $P=>0.005$), except Swedish cells treated with 1µM DHA, 10µM DHA and 0.1µM EPA, which were significantly higher ($F (10, 88) = 9.861$, $P<0.0001$). There was no significant difference in cell viability in the native cells in any PUFA treatment groups.

After 96 hours (Figure d) Swedish cells treated with either 1µM DHA or 10µM DHA still had a significantly increased rate of cell viability across all treatment groups ($F (10, 88) = 9.888$, $P<0.0001$). There was no significant difference between 1µM DHA or 10µM DHA cell viability percentages in Swedish cells ($F (10, 88) = 9.888$, $P=>0.005$). However, at 0.1 µM EPA, there was a significant increase in cell viability in the Swedish cell group compared to the native cell group ($F (10, 88) = 9.888$, $P<0.0001$). Except for cell viability at 1µM DHA, 10µM DHA and 0.1µM EPA, there was no significant difference in cell viability between the native and Swedish cell groups ($F (10, 88) = 9.888$, $P=>0.005$). As well as no significant difference in cell viability in the native cells in any of the PUFA treatment groups, as expected ($F (10, 88) = 9.888$, $P=>0.005$)

Cell Count Analysis: Does PUFA treatment (DHA, EPA, ALA) affect cell viability across both cell groups (SH-SHY5Yswe and SH-SY5Ynative) following 24hr, 48hr, 72hr and 96hr treatment?

As above, Swedish and native cells were treated with three concentrations of DHA, EPA and ALA (0.1µM, 1µM and 10µM) cell viability was measured over 24, 48, 72 and 96 hours by a cell count analysis. Results represent that at all time points in the Swedish cell group, cell viability was significantly higher in the 10µM DHA treatment group. Whereas there was no significant difference in cell viability in the native cell group across all PUFA treatments and time points as cell viability remained constant.

At 24 hours (figure a) Swedish cells treated with 10µM DHA showed significantly higher cell viability than EPA, ALA and 0.1µM DHA ($F (10, 88) = 5.069$, $P<0.0001$). There was also a significant difference in cell viability in cells treated with 1µM DHA compared to cells treated with EPA and ALA ($F (10, 88) = 5.069$, $P<0.0001$). However, the percentage of cell viability of Swedish cells treated with 10µM DHA was significantly higher than that of cells Swedish cells treated with 1µM DHA ($F (10, 88) = 5.069$, $P<0.0001$). There was no significant difference in cell viability found in native cells treated with any concentrations of DHA, EPA, and ALA ($F (10, 88) = 5.069$, $P=>0.005$). However, there was a significant increase in cell viability when Swedish cells were treated with 1µM DHA and 10µM DHA compared to the native group ($F (10, 88) = 5.069$, $P<0.0001$). But no significant difference in cell viability between the Swedish and native cell groups (except Swedish cells treated with 1µM DHA and 10µM DHA) ($F (10, 88) = 5.069$, $P=>0.005$).



<u>Figure 13</u>. Cell count analysis of cell viability of Swedish and native cells post PUFA treatment (DHA, EPA, ALA). Four bar charts produced from cells treated with DHA, EPA and ALA at concentrations of (0.1µM, 1µM and 10µM) at 24 hours (A), at 48 hours (B), at 72 hours (C) and at 96 hours (D). Standard error bars applied to both Native and Swedish cell groups.

At 48 hours (figure b) there was no significant difference in cell viability between Swedish and native groups in any PUFA treatment concentrations (except Swedish cells treated with 1µM DHA and 10µM DHA) ($F (10, 88) = 6.184$, $P<0.0001$). In the native cell group, there was no significant difference in cell viability found between any of the three PUFA treatment groups, as there was no significant effect of PUFA treatment on the native cells ($F (1, 88) = 17.26$, P=>0.005). However, Swedish cells treated with 1µM DHA showed a significant increase in cell viability than EPA and ALA treatment groups ($F (1, 88) = 17.26$, $P<0.0001$). As well as this, the 10µM DHA treatment group showed the highest increase in cell survival, resulting in a significant increase in cell viability compared to all other treatment groups ($F (1, 88) = 17.26$, $P<0.0001$). Swedish cells treated with 10µM DHA had significantly higher cell viability compared to cells treated with 1µM DHA ($F (1, 88) = 17.26$, $P<0.0001$). Swedish cells treated with 1µM DHA and 10µM DHA showed a higher

percentage of cell viability than native cells treated with the same concentrations of DHA ($F (1, 88) = 17.26$, $P<0.0001$).

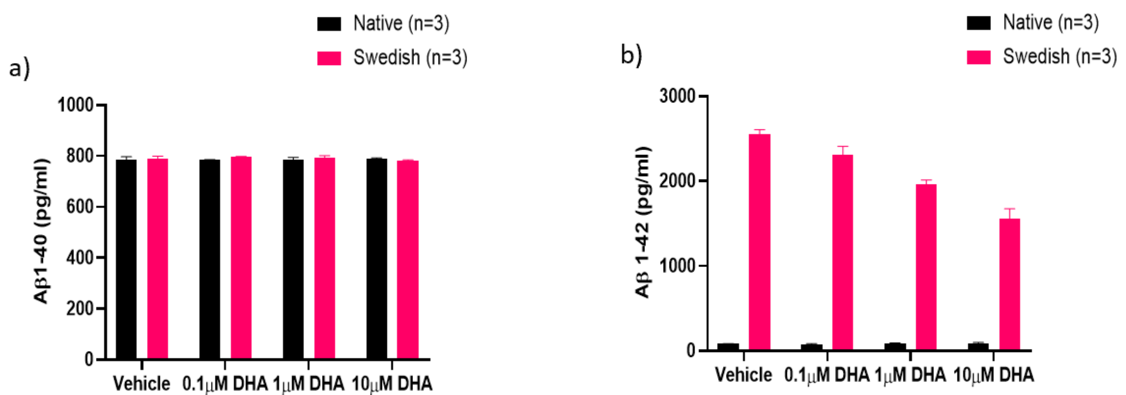
Results show at 72 hours (figure c) there was no significant difference in cell viability in the native cells treated with the three PUFAs at any concentration ($F (1, 88) = 1.940$, $P=0.1671$). As well as this, there was no significant difference in cell survival between native and Swedish cell groups when treated with PUFAs ($F (10, 88) = 10.29$, $P=0.1671$); excluding Swedish cells treated with 10µM DHA. However, in the Swedish cell group, cells treated with 10µM DHA had a significantly increased cell viability than cells treated with 0.1 µM DHA and 1µM DHA ($F (10, 88) = 10.29$, $P<0.0001$). Swedish cells treated with 10µM DHA displayed a significant increase in cell viability compared to cells treated with EPA and ALA ($F (10, 88) = 10.29$, $P<0.0001$). There was no significant difference in cell viability between Swedish cells treated with EPA, ALA, 0.1 µM DHA or 1µM DHA ($F (10, 88) = 10.29$, $P=0.1671$).

96 hours post-PUFA treatment (figure d) SH-SY5Yswe cells treated with 10µM DHA displayed an increase in cell viability, significantly higher than Swedish cells treated with 0.1µM DHA and 1µM DHA ($F (1, 88) = 0.7518$, $P<0.0001$). Results show a significant difference in cell viability between Swedish cells treated with 10µM DHA compared to Swedish cells treated with EPA and ALA ($F (10, 88) = 8.134$, $P<0.0001$). There was no significant difference found in cell viability between Swedish cells treated with either EPA, ALA, 0.1µM DHA or 1µM DHA ($F (10, 88) = 8.812$, $P=0.3883$). There was no significant difference found in cell survival between native cells treated with DHA, EPA or ALA as PUFAs had no significant effect on increasing or decreasing cell viability ($F (10, 88) = 8.134$, $P>0.005$). Results represent that there was no significant difference in cell survival between native and Swedish cell groups when treated with PUFAs $F (10, 88) = 8.812$, $P>0.005$); except Swedish cells treated with 10µM DHA.

Does DHA affect the volume of Aβ 1-40 and Aβ 1-42 produced by Swedish and native cells 48Hrs post DHA treatment?

Native and Swedish cells underwent an ELISA assay to quantify the levels of Aβ 1-40 and Aβ 1-42 being produced after DHA treatment. Cells were treated with three concentrations of DHA (0.1µM, 1µM and 10µM) and an ELISA assay was conducted after 48 hours.

Results from Figure (a) represent DHA had no significant effect on Aβ 1-40 production in either the Swedish or the native cell group at any concentrations ($F (3, 16) = 2.030$, $P=0.1503$).



<u>Figure 14.</u> ELISA assay on SH-SY5Y cells (Swedish and Native) 48 hrs post-DHA treatment, representing volume of Aβ 1-40 and Aβ 1-42 production. A bar chart (a) representing volume of Aβ 1-40 produced by Swedish and native cells 48 hrs after DHA treatment (0.1µM, 1µM and 10µM DHA). A bar chart (b) representing volume of Aβ 1-42 produced by Swedish and native cells 48 hrs after DHA treatment (0.1µM, 1µM and 1µM DHA). Standard error bars applied to native and Swedish cell groups.

However, figure (b) displays DHA had a dose-dependent effect on Aβ 1-42 production in Swedish cells, representing a significant difference between the volume of Aβ 1-42 produced by Swedish and the native cells ($F (3, 16) = 78.64$, $P<0.0001$). At 0.1µM DHA Aβ 1-42 is ≈ 2500 pg/ml and it decreases to ≥ 2000 pg/ml with 1µM DHA and further decreases when treated with 10µM DHA to ≈ 1500 pg/ml. However, DHA at all concentrations had no significant effect on Aβ 1-42 production in native cells, as expected. There was a significant difference in the volume of Aβ 1-42 produced

by Swedish cells treated with 0.1µM DHA and 1µM DHA, as cells treated with 1µM DHA produced a significantly decreased volume of Aβ 1-42 than cells treated with 0.1µM DHA. Similarly, Swedish cells treated with 10µM DHA produced a significantly lower volume of Aβ 1-42 than Swedish cells treated with 0.1µM DHA or 1µM DHA. All three concentrations of DHA treatment (0.1µM DHA, 1µM DHA and 10µM DHA) induced a reduction of Aβ 1-42 produced by Swedish cells compared to the vehicle group.

Discussion

Alzheimer's disease is a neurodegenerative disease with detrimental effects not only on an individual's brain and body but to their quality of life and the people around them (Barbe et al., 2018). Omega-3 fatty acids are essential for healthy brain function, they have been found to benefit health in many ways, as well as being widely researched for various disorders, such as inflammatory disease, diabetes, cardiovascular disease as well as neurodegenerative diseases such as Parkinson's and Alzheimer's(P Ander, MC Dupasquier, A Prociuk and N Pierce, 2003) which inspired this research to be conducted.

SHSY5Y Cell Viability and Aβ Production

Both the Swedish SH-SY5Y and Native SH-SY5Y cells are a clonal population that was derived from neuroblastoma cells in the 1970s. The original cells were cloned three times to produce the SH-SY5Y variation (Ross et al., 1983). The key difference between them is that the native cells are used to model healthy, while the Swedish cells are used to model AD as they have been transfected with the Swedish mutation of AD. This has allowed me to examine the impact of the Swedish mutation on SH-SY5Y cells, including their viability, rate of Aβ40/42 production, and the

effects of DHA, EPA, and ALA treatment. By comparing the results from both cell types, it is clear that any differences can be attributed solely to the mechanisms of the Swedish mutation and AD such as the significantly reduced cell viability of Swedish cells, the increased production of Aβ42 and the improvement in cell viability following Omega-3 treatment.

In these experiments, SH-SY5Y neuroblastoma cells (Swedish mutation and native cells) were first monitored for weeks before treatment to allow us to gain an insight into how effectively the cells survived in the same conditions. Using the MTT assay and cell counts made clear that the viability of the Swedish cells was significantly lower than the native cells (shown in Figure 10).

A Western blot assay conducted on SHSY5Y Swedish mutation cells in previous research showed that the cells produced concentrations of APP at a significantly higher level than the control/non-mutated group. The level of Aβ40/Aβ42 was directly linked to the number of mutations present in the cells, thus resulting in the Swedish mutated cells showing the highest ratio of Aβ40/Aβ42 along with the Indiana mutation (Pahrudin Arrozi et al., 2017). This is consistent with previous research as the Swedish mutated version of SH-SY5Y cells endogenously produce higher levels of Aβ40/Aβ42 compared to native cells (Klein, 2011). Results in this experiment also reflected this, as the Swedish mutated cells endogenously produced a higher concentration of Aβ42 than the native cells allowing them to be great for research due to their versatility.

This study only measured cell death occurring up to 96 hours, and the rate at which cells were dying, shows that if tested for a longer period, it is expected that cell viability would have continued to decrease to lower percentages. When these findings are loosely applied to the true timescale of Alzheimer's disease, where degeneration occurs over decades (even before any symptoms appear), the true percentage of cell death that occurs in the brain of an individual is astounding. It would be interesting if in future research there would be means of quantifying cell death in individuals experiencing AD at different stages, to show the level of degeneration as a

numerical percentage to help with further understanding of the area. It is made clear that the Swedish cells are less viable and more prone to dying from the start, as this occurred from the time they were plated.

It is known that Aβ42 builds up and aggregates into neurotoxic plaques which lead to nerve cell death. As these cells continue to die, the associated brain regions subsequently begin to deteriorate, causing a cognitive decline in individuals (Carter and Lippa, 2001). It is important to remember, as stated above, that it was thought that only the insoluble form of Aβ 42 was harmful, however, it was found that the soluble forms of Aβ 40 are also pathogenic, and can also contribute to synaptic death (Prins and Scheltens,2012). In this research, figure 10 represents the volume of Aβ40 and Aβ42 production in the Native and Swedish cell groups. An ELISA assay was performed to measure levels of Aβ production. The volume of Aβ 40 was produced at a similar rate by the native and Swedish cell groups. However, the volume of Aβ 42 produced in the Swedish cell group was significantly higher than in the native cell group. These findings suggest that, even though small amounts of Aβ 42 are produced by the native cells, the volume of Aβ 42 produced by the Swedish cell group, is significantly higher, and it is produced at a faster rate supporting findings from previous research (Klein, 2011).

This tells us that the mechanisms associated with AD can decrease cell viability and increase the rate of cell death (Brokaw et al., 2020). The native cells were used to represent ‘normal’ neuronal cells, found in healthy brains as a control, it can be conferred that all brains may produce some levels of Aβ 42, and it may aggregate, however, the rate at which this occurs is much slower, and over the lifespan of an individual, it may never reach a volume high enough to have any effects on the function of an individual’s brain. However, in the Swedish group, the cells behave in a way similar to neuronal AD cells found in an individual with AD or at a high risk of developing AD. This cell group showed considerably higher levels of Aβ 42, as well as a faster rate of production which

can be associated with the cognitive impairments commonly found in individuals diagnosed with AD. Although, many other risk factors contribute to the onset of AD and the degeneration of neural cells, such as the presence of Tau neurofibrillary tangles so it is not the aggregation of Aβ alone (Medeiros et al., 2010).

The Effectiveness of DHA, EPA and ALA

DHA, the essential omega-3 fatty acid is known to have neuroprotective properties which have been widely researched and found to be successful (Rapoport & Taha, 2014). In this research the cells were treated with three types of Omega-3 fatty acids; DHA, EPA and ALA, to find out which fatty acid would be the most successful at increasing cell viability (shown in figures 11 and 12). Three concentrations of each Omega-3 fatty were used to also determine the optimal concentrations (0.1µM, 1µM and 10µM).

The results confirmed that DHA was the most successful out of the Omega-3 fatty acids used to treat the cells as the percentage of cell viability continued to increase in the Swedish cell group. The efficacy of DHA treatment was time-dependent, as time increased, so did cell viability, showing that DHA had a significant effect on Swedish cell viability, compared to the control, vehicle, EPA and ALA groups. DHA promoted cell survival in the Swedish cell group most effectively when used in concentrations of 1µM and 10µM, this was shown throughout by results of the cell count analysis, MTT assay and ELISA assay. This was reflected in Figure 13, where both the Native and Swedish cells were treated with various concentrations of DHA, and cell viability in the Swedish mutation cells improved and levels of Aβ42 decreased.

There are a few mechanisms in which DHA is thought to work in the brain which have the potential to impact symptoms related to AD. DHA was found to have an impact on APP processing by upregulating the production of the non-toxic sAPPα from APP cleavage, thus inhibiting and reversing the formation of toxic Aβ42. DHA was shown to increase the use of the non-amyloidogenic pathway in APP processing, rather than the amyloidogenic pathway which is responsible for producing the majority of

Aβ (Heras-Sandoval, Pedraza-Chaverri and Pérez-Rojas, 2016). The results of this experiment support this mechanism of DHA as when the Swe-mutated cells were treated with DHA, the concentration of Aβ42 significantly decreased over the 96-hour timeframe measured, but had little to no effect on levels of Aβ 40.

The results of this experiment could also be explained by the findings of a study on mice models by Yiannopolou et al (2012). It was found that DHA was able to decrease γ and α- secretase activity in the membranes of mice brains. For 4 weeks, mice were fed a DHA- enriched diet or a control diet, following this, levels of α and γ- activity were measured and it was reported that DHA directly decrease the activity of both enzymes. However, is it possible that DHA also acts on the non-amyloidogenic pathway of APP processing, by increasing the use of this pathway to keep levels of Aβ 40 intact (as shown in figures) without the accumulation of Aβ42?

There have been several in-vitro studies which have been supported by this experiment. A study by Hashimoto et al (2011) used SHSY5Y cells infused with Aβ25-35, which were treated with DHA. The results showed that DHA inhibited the neurotoxic effects of Aβ25-35 and improved previous DNA damage caused by Aβ25-35. This highlights the neuroprotective role of DHA against neurodegeneration which occur in diseases such as Alzheimer's and Parkinson's. The results of this experiment showed that when the cells were treated with omega-3 fatty acids (specifically DHA and EPA), there was a significant increase in cell viability, therefore highlighting the neuroprotective role of omega-3 against the toxicity of Aβ. (ML)

Other studies have tested the protective role of DHA and EPA using SHSY5Y cells exposed to Aβ, it was found that both DHA and EPA were effective in improving cell viability and reducing oxidative stress in the cells. On top of this, EPA was found to be the most effective out of the two Omega-3s used as it significantly reduced neuronal cell apoptosis and improved cell viability (Zhang et al., 2018). This was supported by the results of this experiment, as both DHA and EPA were found to

have a positive effect on cell viability and the reduction of Aβ42 at the higher concentrations, although in this experiment DHA was found to be the most effective out of the three.

In contrast, researchers studied the impact of ALA on SHSY5Y cells induced with Aβ25-35 at concentrations of 1, 2.5, 5 and 25 µg/mL were tested. ALA was found to significantly reduce neuronal cell apoptosis and improve cell viability (Lee et al., 2017) which was not supported by the results of this particular experiment. The ALA treatment group was not found to be effective in reducing Aβ42 concentration, nor improving cell viability. However, this could be due to the concentrations of ALA used not being high enough as the maximum concentration used in the research by Lee et al (2017) was 25 µg/mL compared to 10 µg/mL in this research.

Although the findings support the aims and hypothesis of the research, as expected, many questions can be raised. How effective can DHA treatment be in the long term? and will it continue to increase cell survival with time over the real time frame of Alzheimer's disease? This study has shown insight into the potential efficacy of DHA treatment. DHA has been effective over a short time (96 hours). It is expected that it would continue to be effective, however, after some time, beyond 96 hours, cell survival may reach a plateau, which was briefly experienced in these results, as at 96 hours, cell viability was at a similar percentage as it was at the 72 hours checkpoint. Can point can be reached where DHA is no longer further increasing cell survival at an effective rate and maybe higher concentrations of DHA would need to be used.

The use of higher concentrations of DHA could be more effective in decreasing cell death from the beginning which has not been measured in this particular research. The only way for this to be found out is further research into the efficacy of DHA over a long period, with additional concentrations of DHA to be used, or a combination of DHA with either EPA or ALA, which were not as effective alone, but have been found to promote cell survival in combination with DHA (Zhang et al., 2018).

Efficacy of Omega-3 supplements

It is thought that most people receive enough DHA, EPA and ALA through their diet and that blood plasma levels of DHA wouldn't be considered low enough to have an impact unless potentially following a vegan diet (Cunnane et al., 2019). The recommended guideline for omega-3 supplements is no more than 5g of EPA, DHA and ALA a day, as consuming more can result in mild but unpleasant side effects including; nausea, stomach upset and diarrhoea (National Institutes of Health, 2017). It is shown that DHA levels in the blood correlate to levels of DHA intake however, the levels in the blood may not be able to indicate the levels of DHA that reach the brain (Arrellanes et al., 2020). Research showed that blood levels of DHA and EPA increased by 15-20% and ALA by 5% following a 1,000mg omega-3 supplement over 12 weeks. This shows that the omega-3 supplements were effective in increasing blood DHA/EPA and ALA levels, however, it was unclear whether the concentrations of omega-3 achieved were high enough to have a positive impact on someone with AD (Cunnane and Chen, 2015).

Further research on the efficacy of supplements displayed that fish oil supplements were associated with a decreased risk of developing dementia in older and at-risk individuals (Huang et al., 2022). These findings have been supported by several studies whose findings indicated that fish oil intake was associated with a decrease in cognitive decline (Thomas et al., 2020) and a lower risk of developing dementia (Nozaki et al., 2021). This was further supported by results from a meta-analysis of 21 studies that showed that increased fish oil intake was linked to a decreased risk of cognitive decline and development of AD (Zhang et al., 2016).

Results of some smaller clinical trials using omega-3 supplements showed a decrease in cognitive decline in patients in the early stages of AD and the elderly who are at a high risk of developing AD (Arellanes et al., 2020). However, more research is needed as there have been inconsistent results when testing the effectiveness of omega-3, although they may be beneficial in the onset of the disease where brain impairment is mild, and maintain brain function in healthy individuals,

they have not found to be effective for more severe cases and cannot be supported as a treatment of AD (Canhada et al., 2017).

A clinical trial conducted in 2016 by Heras-Sandoval et al, showed that the presence of the APOE4 allele which is a risk factor for developing AD was shown to reduce DHA treatment efficacy in AD patients. Patients were given 2g/day of DHA, and it was concluded that DHA did not affect cognitive impairments or brain atrophy. However, patients with AD who did not possess the ApoE4 allele showed mild positive effects regarding treatment, however, the findings were not significant.

Based on the findings a reason for the supplements not being as effective could be due to higher concentrations of omega-3 being required to have the desired effect. Although research by Cunnane et al (2007) showed that blood plasma DHA levels reflect DHA dietary intake, this does not apply to the levels of DHA or omega-3 that reach the brain. As the increase in levels of blood plasma DHA levels are not proportional to the levels of DHA in the brain, and the levels in the brain are significantly lower. This was backed up by research that showed after 6-months of omega-3 supplement use, participants had a 200% increase of DHA in their blood plasma compared to the placebo, however only a 28% increase of DHA in their CSF, showing that levels in the brain cannot be accurately represented by blood plasma levels (Arrellanes et al., 2020). Therefore supplement doses may need to increase significantly to have a positive effect on the brain or alternative and more effective methods of administering DHA need to be developed.

There are many studies and clinical trials where the use of DHA supplementation has been tested and found to be successful, as well as data from animal models. It is thought that the benefits of DHA alone may be limited as Research from Cole and Frautschy, 2010 suggested that the efficacy of DHA could be increased by combining DHA with other vitamins or antioxidants as their results showed that DHA alone was not sufficient in controlling neuroinflammation and could be improved with the use of an anti-inflammatory agent such as curcumin found in Turmeric.

Overall, it can be concluded that the results support the aims and hypothesis of the experiment and show the neuroprotective role DHA plays in Alzheimer's disease and the potential in slowing cognitive decline in individuals with mild AD or at a high risk of developing the disease.

Conclusion

Alzheimer's disease is a complex neurodegenerative disease affecting a great number of individuals worldwide, and its prevalence in the general population is increasing. Many risk factors have been linked to the onset of the disease, such as age, gender, genetics; a mutation in APP, or a dysfunctional ApoE4, as well as diet: such as omega-3, specifically DHA intake. The role of DHA, EPA and ALA have been examined in this research. It was concluded that DHA was the most effective, followed by EPA in lowering the volume of Aβ 42, promoting cell survival in the Swedish cell mutation group. The effectiveness of DHA has been supported by other researchers but has well been refuted. The mechanisms behind the disease are known, such as the accumulation of Aβ; specifically, Aβ42, which plays a large role in the onset and progression of Alzheimer's as well as the presence of Tau neurofibrillary tangles. Hopefully, with more research, future treatments targeting these specific mechanisms, whether natural compounds such as DHA or PUFA or synthetic molecules could be established. This would allow for better and more effective treatment methods, which could potentially slow down the progression of the disease, instead of treating the cognitive impairments and behavioural symptoms associated with AD, it would improve the quality of life of those living with AD as well as their family/carers and reduce the burden on the NHS.

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APPENDICIES

Appendix a. Raw data for Figure 12. MTT Analysis of PUFA treatment

Appendix b. Raw data for Figure 13. Cell Count Analysis of PUFA treatment