Investigating the potential of human brain tissue and patient fibroblast derived small extracellular vesicles in Alzheimer's disease research

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Abbreviations;

Aβ - Amyloid- β

AD - Alzheimer's disease.

ADL - Activities of daily living

AELN - Autophagic-endolysosomal networks

AICD - Amino-terminal APP intracellular domain.

ALS - Amyotrophic lateral sclerosis.

APP - amyloid precursor protein.

APOEε4 - Apolipoprotein E

BHC - Brain homogenate collagenase

BH - Brain homogenate

BSA - Bovine Serum Albumin

bvFTD - behavioural variant frontotemporal dementia

CBD - Corticobasal degeneration.

CDK5 - Cyclin-dependent kinase

CMA - Chaperone-mediated autophagy.

CMO- Cell Mask Orange

CNS - Central Nervous System.

CBS - Corticobasal syndrome.

CO_2 - Carbon dioxide.

CSF - Cerebrospinal fluid

DDT - Dichloro-diphenyl-tichloroethane

ddH_2O- Double distilled water.

DGCR8- DiGeorge Syndrome Critical Region 8

DMEM- Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic Acid

DPX - Dibutyl Phthalate Xylene.

EV - Extracellular Vesicles.

EOAD - Early-onset Alzheimer's Disease.

FBS -Foetal Bovine Serum

FTD - Frontotemporal Dementia.

FTD-MND- Frontotemporal dementia with Motor Neuron disease.

FTDP-17 - Frontotemporal dementia and Parkinsonism linked to chromosome 17

fNTA- Fluorescent nano-tracking particle analysis.

FUS - Fused in sarcoma.

GPCOG - General Practitioner Assessment of Cognition

GP- General Practitioner

GSK-3β - Glycogen synthase kinase-3

HSPGs - Heparan sulfate proteoglycans.

HSC70 - Heat shock Cognate protein 70

HSP-40 - Heat shock protein 40

HSP-90 - Heat shock protein 90

IADL - Instrumental activities of daily living.

IHC - Immunohistochemistry.

ILVS - Intraluminal Vesicles.

LAMP2A - Lysosome-associated membrane protein, type 2A.

LIR - LC3-interacting region.

LOAD - late-onset Alzheimer's Disease.

MAPT - Microtubule associated protein Tau

MCI - Mild Cognitive Impairment

MEM - Minimum Essential Medium

MiAP - Mitogen- activated protein

miRNA - Micro RNA

MMSE - Mini mental state examination.

MRI - Magnetic Resonance imaging.

MS - multiple sclerosis.

MVBs - Multivesicular bodies.

ND - Neurodegenerative disease/s.

NCL - Neuronal ceroid lipofuscinosis.

NFTs - Neurofibrillary tangles

NTA - Nanoparticle tracking analysis.

PBS - Phosphate buffered saline.

PD - Parkinsons Disease

PET - positron emission tomography.

PrP^{C} - Cellular prion protein.

PPA-FTD - Primary progressive aphasia frontotemporal dementia

PSP-P - Progressive supranuclear palsy syndrome

PS - Pensenilins.

RNA - Ribonucleic Acid

ROS - Reactive oxygen species.

SAP - Stress activated protein.

SEC - Size Exclusion Chromatography.

sEVs - small extracellular vesicles.

SPECT- Single-photon computed tomography.

sv-FTD -Semantic variant frontotemporal dementia

TSG101- Tumor Susceptibility 101.

TREM2 - Triggering receptor expressed on myeloid cells 2.

UBD - Ubiquitin-binding associated domain.

UNC5C - Unc-5 Netrin Receptor C

UPS - Ubiquitin proteasome system

Abstract;

Alzheimer's disease is the most common cause of dementia. Alzheimer's disease is a progressive and irreversible neurodegenerative disease leading on to a decline in cognitive function causing language, memory and behavioural impairment. The two famous pathological hallmarks involved in Alzheimer's disease are the β-amyloid plaques and intraneuronal neurofibrillary tangles. The abnormal protein accumulation increases as the disease progresses with the molecular mechanisms underlying the spread of abnormal protein remaining unclear. Recent research suggests that small extracellular vesicles are involved in the spread of hyperphosphorylated tau and are thought to act as a prion within the brain causing normal healthy tau to become pathogenic tau. Small extracellular vesicles are secreted by all cell types including, brain tissue and they have the ability to pass through the blood brain barrier. This study focuses on exploring the potential use of human patient derived skin fibroblast and human brain tissue derived small extracellular vesicles as a tool to identify dementia biomarkers and to shed light on the disease process. sEVs from human brain tissue and fibroblast were isolated using exo-spin methodology, the sEVs were characterised using immunoblotting and nano-particle tracking analysis. sEV were successfully isolated from human brain tissue from both age-related and Alzheimer's disease cases using CD9, CD63, Flotillin-1 and calnexin antibodies but were not successfully isolated from fibroblast cells. From the whole brain homogenates and sEV fractions further antibodies were explored, hyperphosphorylated tau (AT8) and autophagy marker - p62. The p62 antibody was detected in both samples however, AT8 was only detected in sEVs fractions. Immunohistochemistry was used to further investigate the p62 detection across different braak groups and to explore if there's any positive correlation between the intensity of the stain with the different braak groups. After scoring the slides and dichotomising the data, it was shown the intensity increased as the braak group increased. A chi-squared test deemed the results as significant however, due to lack of samples the results were deemed as unreliable. This study suggests that sEVs can be extracted from human brain tissue and therefore, is able to help further sEV research studies to overcome dementia by using sEVs as a way to differentiate between different disease types.

1.1 Introduction

1.1.1 Dementia

Dementia effects over 50 million people worldwide (Alzheimer's Research UK, 2018) and is a huge socioeconomic challenge. The prevalence of dementia is significantly increasing and is set to nearly triple in the number of cases by 2050 (Ponjoan et al., 2019) due to lack of treatment options. Dementia itself is not a specific disease but is a destructive set of symptoms including memory loss, confusion, difficulty understanding and concentrating, lack of ability to solve problems, repetition of questions or phrases as well as struggling with expressing thoughts or feelings. This causes impairment in a person's cognitive function ability to complete everyday tasks (Duong et al, 2017). Dementia is caused by neurodegenerative diseases such as Alzheimer's Disease (AD) and Frontotemporal Dementia (FTD), which ultimately results in irreversible neuronal death (Niikura et al, 2006). Diseases such as AD and FTD are further characterized by the build-up of abnormal protein accumulation such as; beta amyloid plaques, neurofibrillary tangles (NFTs) and 43 kDa transactive response DNA-binding protein (TDP-43) within the brain, which are known to disrupt numerous physiological processes such as; protein synthesis, protein trafficking, protein degradation and the ubiquitin proteasome system (UPS) (Jellinger, 2010). Dementia related diseases cost over 26 billion pounds per year (Prince et al, 2014). The costs implicated by dementia include; informal care, social care costing and healthcare costs (Prince et al, 2014) and therefore, there is an urgent need to end this global crisis and this is in the hope of researchers. The number of people diagnosed with dementia could nearly triple in the next 31 years resulting in huge economic costs (Ponjoan et al, 2019) due to previous clinical trials not being successful in identifying a disease modifying therapy (Sheinerman et al, 2017) with the exception of the recent Lecanemab trial (van Dyck et al, 2023). This drug trial suggests that the drug Lecanemab can therapeutically target β-Amyloid plaques (caused by accumulation of Amyloid-β protein) and reduces the AD associated cognitive decline (van Dyck et al, 2023). The β-Amyloid plaques are thought to be one of the underlying drivers of AD and therefore, is a key focus for a therapeutic intervention (van Dyck et al, 2023). However, β-Amyloid is not the primary driver of other

neurodegenerative disease such as FTD, and so other therapeutic targets and diagnostic markers are still needed to help in other types of dementia.

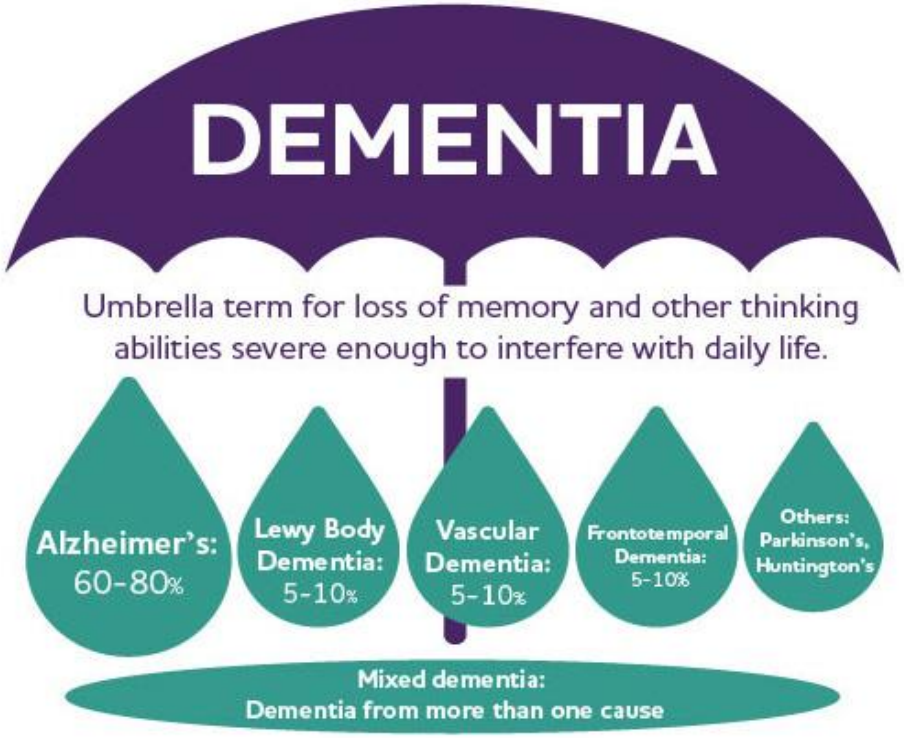


Figure 1; Dementia is an umbrella term for the cognitive impairment caused by neurodegenerative diseases; Alzheimer's diseases is a neurodegenerative disease that represents up to 80% of people diagnosed with dementia, other types of dementia include vascular dementia, Dementia with Lewy bodies and frontotemporal dementia. When a person is diagnosed with more than one type of dementia for example; Alzheimer's disease and Vascular dementia it is known as mixed dementia which is very common (ARUK, dementia statistics hub, 2018).

1.1.2 Types of Dementia and their prevalence

As mentioned previously, the term dementia is used to describe a set of symptoms associated with impaired cognitive ability and physiological functioning which is progressive and fatal overtime. Neurodegenerative diseases which induce dementia include; AD, FTD, Vascular dementia (VaD) and dementia with Lewy bodies (DLB) are the four most common types of dementia as shown in figure 1 (Alzheimer's Research UK, 2018).

Neurodegenerative diseases are the main cause of dementia, the most common type of disease is AD, accounting for 62% of dementia diagnosis. VaD is the second most common type of dementia accounting for up to 17% of cases, mixed dementia accounts for 10% of cases, DLB & FTD are the least common types accounting for 5-10% of the overall dementia cases (Prince et al, 2014). The exact number of people diagnosed the different types of dementia are stated in figure 2.

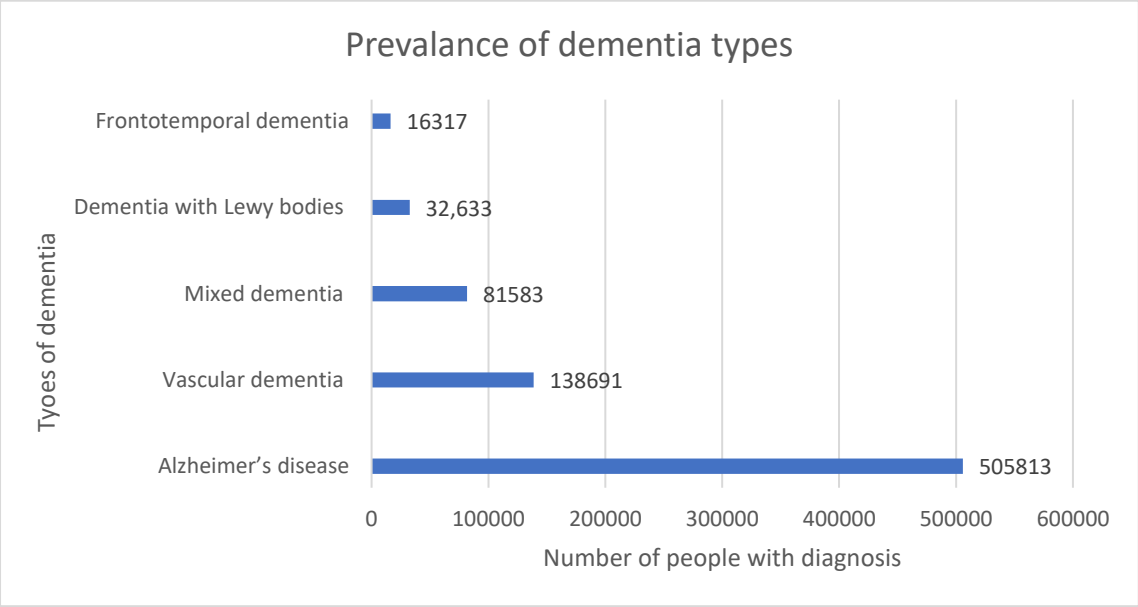


Figure 2; The number of people diagnosed with the common causes of dementia in 2014 (edited from Prince et al, 2014).

1.1.3 Pathogenesis of neurodegenerative disease

Neurodegenerative diseases are pathologically characterised by the build-up of abnormal protein within the brain, which has been shown to contribute to synaptic dysfunction and neuronal death (Weller & Budson, 2018). These proteinacious accumulations include β-amyloid plaque deposition and neurofibrillary tangles (NFTs) of hyperphosphorylated tau as well as 43 kDa transactive response DNA-binding protein (TDP-43) and fused in sarcoma (FUS) (Lashey et al, 2015). Different neurodegenerative diseases show different neuropathological changes despite them all leading onto dementia, with these changes impacting different brain regions and ultimately resulting in different clinical symptoms (Duong et al, 2017). According to previous studies, the accumulation of abnormal proteinaceous deposits occurs decades prior to symptom manifestation for example, the β-

amyloid plaques associated with AD are known to accumulate at least 20 years prior to experiencing any disease symptoms (Mormino & Papp, 2018). Additionally, the concentration of tau protein in cerebrospinal fluid (CSF) was found to be increased 15 years prior to the patient experiencing any onset symptoms and brain atrophy has also been evidenced 15 years prior to symptom presentation (Bateman et al, 2012). Additionally, FTD studies show that grey matter changes can be observed via MRI at least 10 years before any symptoms are shown and white matter abnormalities are seen even earlier (Greaves &Rohrer, 2019). Given these neuropathological changes precede symptom presentation, early detection methods to identify disease drivers and early therapeutic intervention is a significant focus of dementia research, along with developing novel methods to identify early disease biomarkers.

1.2 Symptoms

AD is mainly associated with memory loss, confusion and repetition during the earlier stages (Weller & Budson, 2018) however, during disease progression the symptoms become more significant including wandering behaviours, changes in personality, mobility issues, delusions, communication disturbances, inability to complete daily tasks, incontinence and dysphagia (Hildreth & Church, 2015). In the later stages of disease, AD patients may require palliative care (end of life) where everything in the body operates more slowly (Litcher & Hunt, 1990).

FTD can be divided into 3 clinical subtypes characterised by different symptoms. Behavioural variant frontotemporal dementia (bvFTD) - display a set of behavioural symptoms including disinhibition, apathy, abnormal eating habits (such as developing a sweet tooth or exhibiting hoarding), loss of empathy and obsessive-compulsive behaviour. Patients with primary progressive aphasia frontotemporal dementia (PPA-FTD) develop agrammatism and/or apraxia of speech whilst semantic variant frontotemporal dementia (svFTD) patients develop anomia, prosopagnosia and impaired single word comprehension (Lashey, Rohrer, Mead & Revesz, 2015). FTD patients show behavioural changes in the first instance but may also experience the more common dementia signs such as memory loss,

confusion and verbal repetition. However, each dementia patient may experience different symptoms and symptoms may vary from day to day therefore, the symptoms shown via dementia patients are not used alone to make a diagnosis and further tests need to be carried out (Mohandas & Rajmohan, 2009 & Weller & Budson, 2018) before making a diagnosis.

People with FTD may also develop motor deficits and present in association with other neurodegenerative diseases such as amyotrophic lateral sclerosis (FTD-ALS) or Parkinson's disease. FTD can also be present with corticobasal syndrome (CBS), symptoms of which include, problems with movement, memory and language or progressive supranuclear palsy (PSP) which is associated with balance problems, muscle stiffness, speech problems and difficulty swallowing (Greaves &Rohrer, 2019). These neurodegenerative diseases will be described in more detail in the 'Diseases relating to FTD' chapter, but it should be noted that the clinical and pathological heterogeneity of these disorders has contributed to the challenge in both treatment development and early diagnosis.

1.3 Alzheimer's Disease

AD is the most common type of dementia accounting for up to 70%-80% of cases (Reitz & Mayeux, 2014) and the sixth leading cause of death in the United States and the fifth leading cause of death world-wide (Jutkowitz et al, 2017). AD can be split into 2 subtypes, early-onset AD (EOAD) and late-onset AD (LOAD).

On November 4, 1906 the German clinical psychiatrist and neuroanatomist, Alois Alzheimer, gave a lecture in which described AD for the first time as a form of dementia as well as describing his patient Auguste Deter symptoms (Maurer et al, 1997). The 51-year-old female from Frankfurt experienced a progressive cognitive impairment overtime and after death, there were plaques and neurofibrillary tangles found within the brain tissue. The name Alzheimer's disease was named after Alois Alzheimer (Maurer et al, 1997).

The pathological features seen in 1906 discovered by Alzheimer are the two most famous pathological hallmarks still today. The plaques identified are now known to compose of Aβ1-42 peptide and the NFTs are now known to contain hyperphosphorylated microtubule-associated protein tau; *MAPT* (Miao et al, 2019)

The disease progression and the number of NFTs present within the brain has a positive correlation with cognitive impairment, and despite Aβ plaques being a key pathological hallmark of AD there is a less significant correlation with disease progression when compared to tau accumulation (as shown in figure 3) (Braak H & Braak E, 1991, Murphy & LeVine 2010). Neurofibrillary changes also occur in different forms including neuritic plaques, neurofibrillary tangles and neuropil threads (as shown in figure 4). The localisation of NFTs and neuropil threads have been associated with the severity of disease and been characterised into stages known as braak staging (Braak H & Braak E, 1991). The braak staging system works on the localisation of tau within the brain regions; stages 1 and 2 involve mild alterations within the transentorhinal cortex, stages 3 and 4 often relates to mild cognitive impairment (MCI) or early disease, and show tau present in the transentorhinal cortex and entorhinal cortex proper whereas stages 5 and 6 are related to established/severe AD with a destruction of virtually all isocortical association areas (Braak H & Braak E, 1991). Pathogenic tau has been shown to spread between cells and regions using neuronal connections and is thought to spread by acting in a 'prion' manner causing healthy tau to become pathogenic (Lace et al, 2009, Zhang et al, 2021). The precise mechanisms of tau pathology propagation still remain unclear (Zhang et al, 2021), however, a recent study suggests a role of small extracellular vesicles (sEVs) in this spread, progradation and seeding of abnormal tau protein (Ruan, 2022). This hypothesis will be discussed further in the chapter 'sEVs & Dementia'.

1.3.1 Early-onset Alzheimer's Disease

EOAD is = diagnosed in individuals under the age of 65 and is the most common cause of early onset dementia and accounts for up to one third of patients with young onset

dementia (Mendez, 2017). EOAD is known to have a bigger tau burden, more white matter abnormalities as well as having a greater genetic pre-disposition (Mendez, 2017) and the symptoms progress quicker in this form of disease. Patients with EOAD are reported to initially have better memory recognition scores and semantic memory but have difficulties with language, executive functions, visuospatial skills and attention compared to patients with LOAD (Joubert et al, 2016).

There are many mutations within the amyloid precursor protein (*APP)* and presenilin 1 and 2 *(PSEN1 and PSEN2)* that thought to be the pathogenic driver of EOAD. EOAD accounts for roughly 5% of all AD cases (Giau et al, 2019). *APP* mutations were observed to be present in less than 1% of EOAD patients, *PSEN2* mutations were observed in 1% of EOAD patients and *PSEN1* mutations have been observed in 6% of EOAD patients (Brouwers et al, 2008).

1.3.2 Late-onset Alzheimer's Disease

LOAD is the most common type of AD accounting for 90-95% of all AD diagnosis affecting people aged 65 and above. LOAD is thought to be more associated with environmental risk factors than genetic risk factors. This risk factors include; hypertension, smoking, diabetes, depression, insomnia and heart disease (Rabinovici, 2019). The most complex genetic risk factor associated with LOAD is the human apolipoprotein E (*APOE)* gene which exists in 3 polymorphic alleles; ε2, ε3, ε4 (Liu et al, 2013). The *APOE* ε4 allele is the greatest risk factor in LOAD (Van der filer, 2011). The *APOE* ε4 allele is the most associated genetic risk of developing AD and this has attributed to the enhanced protein aggregation, a decrease in the clearance of Aβ peptide, the increase in levels of hyperphosphorylated tau, reduction of glucose metabolism and impaired vascular function (Liu et al, 2013). The prevalence of the *APOE* gene alleles are; Ɛ2(8.4%), Ɛ3(77.9%) and Ɛ4(13.4%) (Liu et al, 2013). In addition, triggering receptor expressed on myeloid cells 2 (*TREM2*) and Unc-5 Netrin Receptor C (*UNC5C*) mutations are also thought to be related risk of LOAD but not EOAD, with their molecular contribution to LOAD manifestation still being not fully understood (Ridge et al, 2016).

1.4 Frontotemporal Dementia

Despite FTD being the less common type of dementia, FTD is the second most common form of early onset dementia with AD being the most common form of early onset dementia (Onyike & Diehl-Schmid, 2013). FTD is accounts for 5-10% of dementia cases (Snowden et al, 2002). FTD was first discovered in 1982 and was first known as Pick's disease (Pick, 1892 & Alzheimer, 1991). Sv-FTD is present in up to 20-25% of FTD cases, PPA-FTD is also present in up to 25% of cases meaning bvFTD is the most common accounting for up to 50% of all FTD cases ((Johnson et al, 2005). FTD progresses significantly; the survival time for bvFTD is around 6 years (Hodges et al, 2003) whereas the other two subtypes have a much longer survival rate; SD; 11-12 years & PPA-FTD; 9 years from symptom onset (Roberson et al, 2005). Patients who suffer from FTD are often associated with motor neuron disease (FTD-MND) which has an even shorter survival rate of around 3 years (Josephs et al, 2005). Survival rates will depend upon the person as well as other medical/ clinical factors (Hodges et al, 2003). Currently, there are no disease modifying therapies associated with FTD but there are symptomatic treatments available. Each of the clinical subtype show different pathologies at post-mortem - the presence of tauopathies within subtypes is shown in figure 4.

FTD is a spectrum disorder which overlaps with other neurodegenerative diseases sharing similar symptoms and pathology. These disorders include frontotemporal dementia with motor neuron disease (FTD-MND), progressive supranuclear palsy syndrome (PSP-S) and corticobasal syndrome (CBS) (Olney et al, 2017).

1.4.1 Diseases relating to FTD

PSP-S is also known as Parkinson-plus disorder. PSP-S is an uncommon neurological disorder which shows similar symptoms to those shown in late bvFTD including; difficulty with swallowing, impaired vision and eye movements as well as balance, behaviour, mood and cognitive disturbances (Steele et al, 1964). PSP-S is linked with the severity of abnormal tau accumulation within different brain regions causing neuronal death (Dickson et al, 2010).

The cause of PSP-S still remains unknown, although there are environmental factors such as toxins which are thought to influence the cause of disease (Golbe et al, 1998, Rampello et al, 2005).

CBS or also known as Corticobasal Degeneration (CBD) is a sporadic neurogenerative disease with overlaps of FTD symptoms and pathology. CBD is progressive disease with symptoms such asapraxia and aphasia, which are common symptoms seen in PNFA- FTD (Dickson et al, 2002). CBD does not have a specific clinical phenotype as CBD can show a wide range of clinical presentations. Histological examination of brain tissue shows neuronal loss and ballooned type neurons as well as the presence of tau aggregates within neurons and astrocytes (Dickson et al, 2002). The symptoms, pathology and regions affected are similar to those seen in PNFTA-FTD.

Amyotrophic lateral sclerosis (ALS) is the most common type of motor neuron disease. ALS/MND may develop in patients with bvFTD, and this overlap is observable genetically, pathologically and clinically (Phukan et al, 2007). A positive pathology result of ubiquitin/ TDP-43 inclusions are seen in patients with ALS within the spinal cord and motor cortex, which is also seen in patients with FTD (Neumann et al, 2006). Patients who receive a diagnosis of FTD followed by MND are given a diagnosis of FTD-MND however, patients who develop ALS before dementia are given a diagnosis of amyotrophic lateral sclerosis frontotemporal dementia (ALS-FTD). The difference between the two disorders is that FTD-MND has a predominantly cognitive symptom onset whereas ALS-D has a predominantly motor symptom onset (Josephs et al, 2005).

1.4.2 Pathological proteins in AD and FTD

1.4.2.1 Tau

Tau is one of the proteins associated with AD and can be found within a characteristic lesions known as an NFT (Miao et al 2019). Under physiological conditions tau is a microtubule associated protein which normally functions to stabilise neuronal microtubules (Moloney, Lowe, Murray, 2021). However, in pathological conditions the tau protein may

undergo post-translation modification mainly due to phosphorylation which can result in aggregates of insoluble tau protein known as NFTs, confirmed to be toxic to neurons and other cell types. Tau aggregations are known to cause a number of neurological disorders known as tauopathies (Jiang & Bhaskar, 2020).

The interest in tau protein began when various altered proportions of the various tau isoforms were observed in frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Mutreja, Combs & Gamblin, 2019). The human tau gene is located chromosome 17 (17q21) and consists of 16 exons (Neve et al, 1986). The tau gene encodes for six different isoforms within the adult human brain which are produced via alternative splicing of exons, 2, 3 and 10 creating isoforms with 352-441 amino acids (Lace et al, 2007). The six different tau isoforms all differ from each other depending on the presence and absence of inserts (29 or 58 amino acids) in the N-terminus and the presence of 3 or 4 repeats domains in the C-terminus (Goode & Feinstein, 1994). Each of the six tau isoforms are differentially expressed during development therefore, each isoform potentially has its own particular physiological role (Lace et al, 2007). In a healthy brain the *MAPT* protein promotes the formation of axonal tubules, stabilises them and derives neurite growth and therefore, the dysregulation of tau affects the microtubule complex, leading to tau detachment, causes instability as well as perturbs axonal transport (Hong et al, 1998). Microtubules have a tube-shape structure which allows nutrients and other substances to pass through and reach other parts of the neurons. However, in AD the aggregate tau builds up and causes the internal skeleton to fall apart which correlates to neuronal death and cognitive decline seen in dementia patients (Lee et al, 1991 & Von Bergen et al 2000).

The microtubule binding is regulated by the number of binding repeats as well as the phosphorylation state of the protein, with phosphorylation resulting microtubule detachment (Dixit et al, 2021). In AD, tau becomes abnormally hyperphosphorylated which results in aggregates of tau accumulating to form NFTs (Buée et al, 2000). Abnormal hyperphosphorylation of tau is known to be initiated in a number of physiological scenarios, including oxidative stress, imbalance of tau kinases and tau phosphatases as well as impaired glucose metabolism (Arnold et al, 1996 & Liu et al, 2004).

Protein kinases including; glycogen synthase kinase-3 (GSK-3β), cyclin-dependent kinase-5 (cdk5), stress-activated protein (SAP) kinase & mitogen- activated protein (MiAP) kinases have shown to initiate tau hyperphosphorylation (Baudier et al, 1987, Correas et al, 1992 & Drewes et al, 1992). Alterations in protein kinase expression levels are known to activate certain tauopathies which could alter tau phosphorylation leading to neurodegeneration. For example, GSK-3β has been found in NFTs in AD and is also present in glial and neuronal cells, tau deposits in other neurodegenerative diseases including PSP, PD and CBD (Pei et al, 1999 & Ferrer et al, 2002). MiAP, SAP, p35 & p25 (which are needed for cyclin-dependent kinase-5 activation) expression levels are increased in AD brains (Tseng et al 2002). Therefore, this evidence suggests how important it is to have a strict balance between the phosphorylation and dephosphorylation of tau.

The adult human brain expresses all six isoforms and are characterised as 3R or 4R dependent on whether they contain 3 or 4 carboxyl terminal repeat domains (Lee, Cowan & Kirschner, 1988). The brain should contain an equal amount of 3R and 4R tau isoforms. However, mutations in the *MAPT* gene can disrupt the balance of 3R and 4R triggering pathological tau aggregation and NFT formation (Goedert et al, 1989). There are other neurodegenerative diseases associated with the imbalance of 3R and 4R isoforms of tau, 3R tau isoforms are seen in the Picks diseases associated lesions whereas 4R tau isoform containing lesions are seen in the following diseases; CBD, PSP and Argyrophilic grain disease (Bahia, Takada & Deramecourt, 2013). The lesions of AD contain both 3R and 4R tau isoforms (Cherry et al, 2020).

In pathological conditions the tau protein undergoes other post-translational modifications including glycosylation, glycation, phosphorylation, nitration and aggregation (Gong & Iqbal, 2008) which also causes tau to detach from the microtubule and can encourage the formation of aggerates & NFTs inside the neurons and glial cells (Ingelsson et al, 2004). Tau accumulation has a positive correlation with disease progression and braak staging (Braak H & Braak E, 1991).

The Braak stage system describes the localisation of tau present within brain at different stages of disease (see figure 3). It is known that as AD progresses into different stages of

disease the amount of NFTs also increase and spread into other brain regions in a sequential way that aligns with neuroanatomical circuitry (Lace et al, 2007) suggesting a cell to cell propagation of the disease.

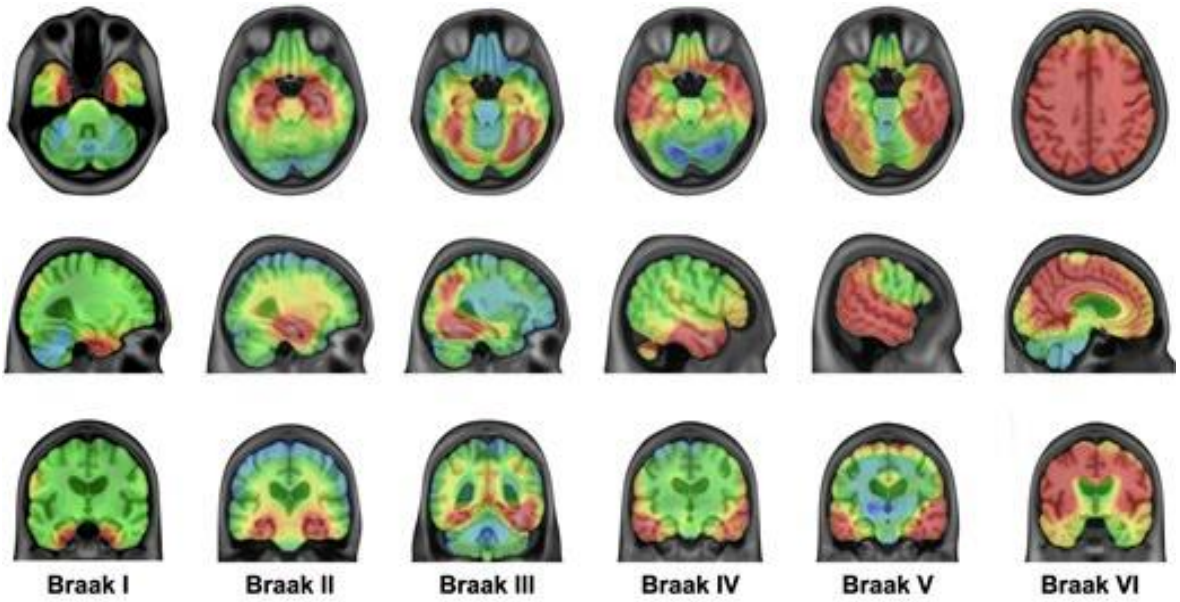


Figure 3; An image to show the amount of NFTs present within the brain at different stages of the braak group system. The NFTs are represented using the colour red, as the braak group increases from a controlled brain scan to Alzheimer's the amount of NFTs increase but also shows how the disease progresses more of the brain becomes damaged from the early stage to the later stage. This image is also evidence that tau spreads between brain sections and regions as the disease progresses. It is expected that all regions of the brain are expected by NFTs in the final stage (Pascoal et al, 2020). This shows a positive correlation between the amount of tau present with disease progression.

Understanding the mechanisms underlying this spread of disease is important to development therapeutic interventions to interfere with disease progression. Additionally, understanding 'what' is being spread could enable the identification of biomarkers to help diagnostically differentiate between the different types of dementia associated disease. It is important to understand where the disease initiates in the different forms of dementia, so to enable the development of targeted treatment strategies. Understanding the nature of this spread would also allow us monitor disease progression and monitor whether which therapeutic agents could be useful or not.

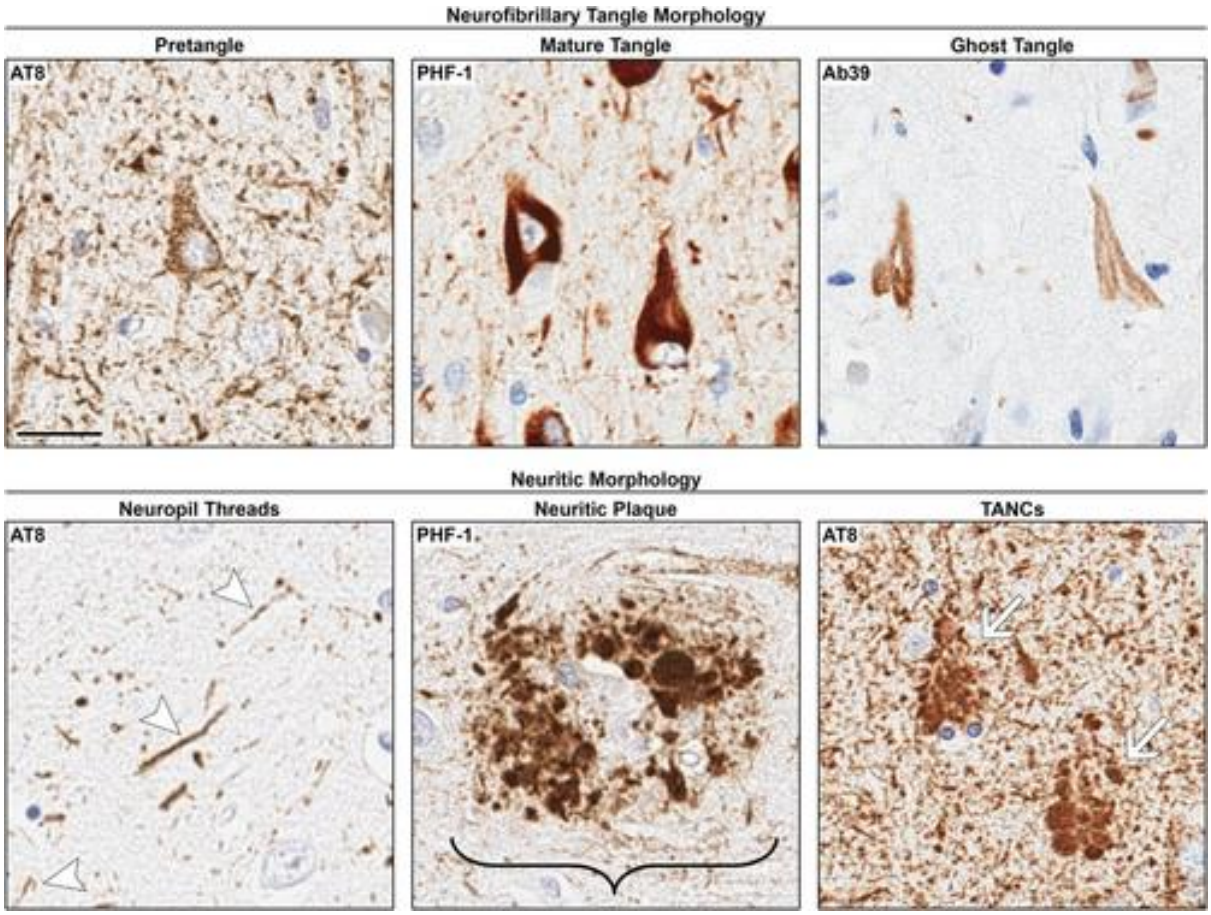


Figure 4; A haematoxylin counterstain to show the present of the different tau pathologies present in AD within the CA1 subsector of the hippocampus. The scale bar measures 25 μm (Moloney, Lowe, Murray, 2021).

There is thought to be over 20 different tauopathies that affect different brain regions, displaying different clinical symptoms and result in different types of dementia (Williams, 2006). Researchers are desperate to identify biomarkers to distinguish between the different types of dementia associated with the different tauopathies, so targeted treatment methods can be developed. Due to there being many different types of tau and disease driving proteins including beta-amyloid, alpha-synuclein and TDP-43, the treatment methods for different types of dementia may need to vary to target the specific driving pathogenic protein species (Jiang & Bhaskar, 2020). Investigating different tauopathies and understanding which tauopathies are related to which types of disease is important to diagnosis, create biomarkers, give a prognosis and create therapeutic inventions by understanding the clinical and molecular differences of tauopathies and how they spread from region to region.

1.4.2.2 Aβ

The human amyloid precursor protein *(APP)* gene is located on chromosome 21 containing at least 18 exons (Yoshikai et al, 1990) and is a type 1 membrane glycoprotein mainly known for its pathogenic contribution in AD (Daigle & Li, 1993). The *APP* gene is involved in a number of physiological processes including; nervous system function and development, as well as synapse formation and function (Aydin, Weyer & Müller, 2012). A number of *APP* cleavage products are thought to be a main contributor in AD causing synaptic dysfunction, cognitive decline and plaque deposits containing the β-amyloid peptide (Goldgaber et al, 1987). Human *APP* can be processed as part of two pathways the amyloidogenic pathway and nonamyloidogenic pathway. *APP* is first cleaved by α-secretase (nonamyloidogenic pathway- normal pathway) or β-secretase (amyloidogenic pathway- pathological pathway), the ladder leading to the generation of a more toxic protein fragment (de Paula et al, 2009). The structure of both pathways is shown in figure 5. The β-secretase pathway is the pathway known to be involved in AD, causing the extracellular amyloid deposits & plaques through the generation of γ-Secretase cleavage of CTF83 and CTF99 which leads to the generation of p3 and Aβ, as well as the amino-terminal APP intracellular domain (AICD) (Chow et al, 2010).

The most popular historic hypothesis surrounding AD is that the β-amyloid peptide (Aβ1-42) is the main cause. In 1984, Aβ was partially isolated from meningeal vessels of AD cases and obtained a partial amino acid sequence. It was known that people with Down syndrome have a significantly increased risk of AD and later that year an amino acid sequence was obtained from a down's case realising it was the same amino acid sequence (See review, Hardy, 2017). This evidence shown that Down syndromes could be a potential model for researching AD as well as suggesting that, the genetic defect is also in AD was located on chromosome 21 (See review, Hardy, 2017). In the first instance, Glenner thought that overproduction of Aβ lead onto AD. After other groups managed to clone the *APP* gene, Glenner first predication of the location of *APP* was correct, it was located on chromosome 21 (See review, Hardy, 2017). After genetic analysis and gene cloning it was found that the Alzheimer locus was also on chromosome 21 and was positioned not that far away from *APP*

gene. After multiple research groups on the race to find the directly link between *APP* and AD many errors were made along the way (See review, Hardy, 2017). However, in 1990 John Hardys group found the first *APP* mutation *APP*V717I using a PCR sequencing machine which later that year two more mutations were found relating to the *APP* gene. *APP*V71G was the second mutation found at the same codon, the third mutation was also found in the same position and this therefore, this was the first molecularly defined the causes of AD (see review, Hardy, 2017).

There are two major forms of Aβ in the brain, Aβ40 and Aβ42 with Aβ42 differing from Aβ40 by only 2 residues. This form is known to be much more prone to aggregation and more toxic than Aβ40 (Philips, 2019). Furthermore, Aβ42 is known to aggregate faster than Aβ40 (Yan & Wang 2006). Aβ42 is form of Aβ species found in the parenchymal senile plaques In AD brains (Yan & Wang 2006). Evidence shows that oligomeric Aβ is also a harmful and toxic form of Aβ, which is generated both extracellularly and intracellularly (Reiss et al, 2018). Aβ42 is known to be toxic to neurons and disruptive to many physiological processes resulting in the leakage of ions, disruption of cellular calcium balance, and loss of membrane potential. Aβ42 can also promote apoptosis which contributes synaptic loss, and disrupt the cytoskeleton (Reiss et al, 2018). However, from pre and post-mortem brains of patients with AD, it shows a limited correlation with the degree of impairment and the amount of Aβ present in the cortex when compared to tau accumulation (Pike et al, 2007) however, this protein is clearly toxic to cells *in vitro* (Fontana et al, 2020). The highly insoluble Aβ plaques need to be present to make a diagnosis for AD post-mortem. Understanding the types of Aβ present (soluble, aggregates, oligomeric) through biomarker detection would allow targeted therapeutic approaches towards these damaging proteins. Accurate disease biomarkers would also allow more robust monitoring of disease progression as well as monitoring the success of treatment methods.

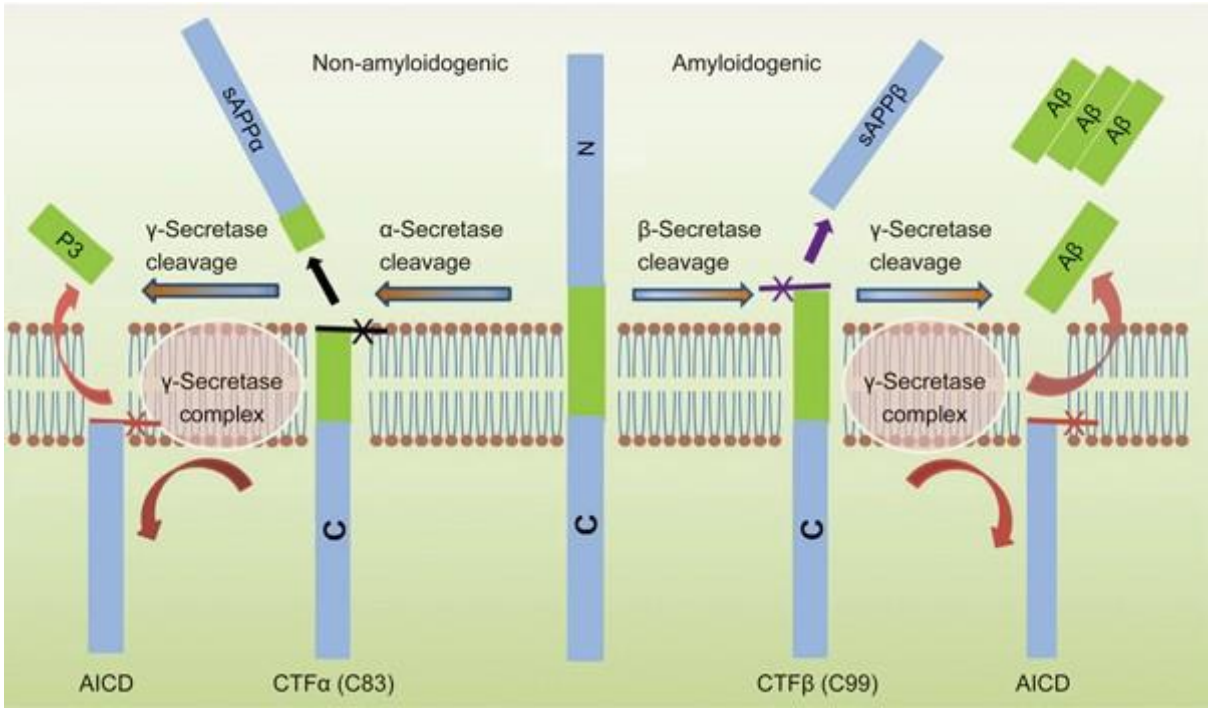


Figure 5; The non-amyloidogenic and Amyloidogenic pathway of Amyloid beta. The β-secretase pathway is the amyloidogenic pathway, which is known to be the pathway involved in Alzheimer's Disease as well as other neurodegenerative diseases (Chen et al, 2017).

1.5 Diagnosis of AD & FTD;

Dementia related diseases such as; AD and FTD are diagnosed based upon the clinical presentation to fulfil several diagnostic criteria as well as a study of the patients bodily fluid samples and neuroimaging assessment (Weller & Budson, 2018). The only definitive diagnosis of AD and FTD requires post-mortem evaluation of brain tissue which is not an effective diagnostic method to those living patients. The diagnostic process for AD and FTD includes ruling out other systemic causes of dementia and involves blood tests to check liver, kidney and thyroid function as well as vitamin B12 levels, haemoglobin A1C levels and urine analysis. There are multiple cognitive assessments used to check a person's mental abilities including; the General Practitioner Assessment of Cognition (GPCOG), assessment of daily living and mini mental state examination (MMSE). The GPCOG will test a patient's memory and cognition. Assessment of daily living is an assessment split up into 2 main categories; basic activities of daily living (ADL) and instrumental activities of daily living (IADL). Basic activities of daily living include fundamental skills learnt early on in life including; personal hygiene, dressing, eating and toileting (Boyle et al, 2002). Basic ADL

often decline in the much later stages of dementia however, IADL skills relate to more complex activities including; managing finances and medication. Impairments of IADL are more present in MCI and early dementia (Cahn-Weiner et al, 2007). It is known that as cognition does decrease overtime as dementia becomes more severe (Helvik et al, 2014) and therefore, the outcome of the assessments can determine whether a patient is struggling with cognitive functions or if there's a possibility of it being a dementia related disease based on the known information. The MMSE is the most common and best-known assessment used, is it a short screening assessment that consists of 30 questions to test the cognitive ability of a patient, this test investigates a patient's attention span, memory, language and orientation (Diniz et al, 2007). The test is scored, the test has a cut off traditionally of 23/24 with suspected impairment or dementia (Diniz et al, 2007). FTD patients experience behavioural changes with the decline in cognitive ability appearing much later therefore, the cognitive test for diagnosing FTD may not be appropriate until later stages of disease (Mohandas & Rajmohan, 2009).

Brain scans are also used as part of a wider assessment to diagnosis dementia, brain scans alone cannot diagnosis dementia. A magnetic resonance imaging (MRI) scan is used to explore blood vessel damage that happens in vascular dementia and signs of strokes which is a main contributor of vascular dementia. An MRI scan can also show the atrophy of brain regions for example; atrophy of frontal and temporal in relation to FTD, or temporal lobe atrophy in AD (NHS, 2020). Analysis of specific tau and amyloid species in the cerebrospinal fluid (CSF) has also shown to be useful in diagnostics but this is an invasive approach. Due to imaging methods such as positron emission tomography (PET) being a less invasive but a more expensive approach (Budson & Solomon, 2012). CSF samples are obtained via lumbar puncture and are used to observe the presence of Aβ42, hyperphosphorylated tau peptide (p-tau), and total tau protein content. This test is more invasive than PET imaging and this is significant given the cognitive status of the dementia patient, but the method costs less (Hampel et al, 2008). This method is also less accurate than PET (85-90% accurate), carries more risks (such as infection) and often takes weeks to obtain results compared to the PET imaging (Palmqvist et al, 2015). PET scan is used to detect the presence of Aβ plaques caused by Aβ1-42 peptides within the brain of living patients, this method has up to 96% sensitivity and 100 specificity proved later via autopsy (Saint-Aubert, 2013) however, this

method is expensive (Budson & Solomon, 2012). Therefore, there is a need for a much cheaper but a more accurate and a less invasive biomarkers that not only sheds a light on the degree of abnormal protein accumulation, but also on the early drivers of disease pathogenesis. Biomarkers to allow earlier diagnosis, in the very early stages of brain alteration and prior to symptom development will help identify individuals would could benefit from preventative treatment administration.

Research studies are also focussing on improving the sensitivity of biomarkers, as well as identifying new biomarkers for use and in particular, a set of biomarkers that could allow an accurate differentiation between the different dementia types (Dong et al 2020). Given the implication of sEVs in the spread of AD and FTD pathology, sEV cargoes could provide a novel source of such biomarkers, but in the first instance, these need to be successfully purified from a range of tissues, if these studies are to be implemented. This will be explored further in the chapter 'sEVs as a biomarker and therapeutic'.

1.6 FTD VS AD

Whilst FTD and AD share some symptoms, they are clinically heterogenous and are also pathologically different with respect to the presence of proteinaceous lesion and also have different disease driving genetic mutations.

FTD and AD can both present with accumulation of hyperphosphorylated tau which can manifest in a variety of lesions including neurons, astrocytes, microglia and oligodendrocytes (Ballatore, Lee & Trojanowski, 2007). In AD, the majority of the tau pathology presence is neuronal, whereas within other neurodegenerative diseases, the presence of glial tau is identified post-mortem (Feany & Dickson, 1996). The majority of the glial tau is found in astrocytes and oligodendrocytes (Ghoshal et al, 2001) but can also be seen in microglial cells (Ghoshal et al, 2001) and this pathology is variable in different types of FTD (Lashley et al, 2015). Furthermore, the presence of tau within different lesions is used to differentiate between different neurodegenerative diseases. Tau pathology present within oligodendrocytes vary in size and morphology across different tauopathies. These

lesions are largely tau-positive cytoplasmic inclusions known as coiled bodies, and are associated with the following diseases; PSP, CBD and Pick's disease (PiD) (Arima, 2006). The morphology of the FTD pick bodies associated with these diseases are shown in figure 6, which are associated with FTD subtypes; bvFTD & PPA-FTD which are characterised by the presence of Pick bodies, inclusions composed primarily of 3R (repeated) tau (Morris et al, 2002). Another type of tau pathology present within oligodendrocytes are argyrophilic threads also associated with PSP and CBD (Arima, 2006). Astrocytic tau pathology is associated with argyrophilic grain disease (AGD), PSP and PiD (Leyns & Holtzman, 2017). The presence of tau-positive glial inclusions has been suggested to be distinctive for different neurodegenerative diseases and have distinct profiles however, their biochemical characteristics and role in disease is still not fully understood (Leyns & Holtzman, 2017).

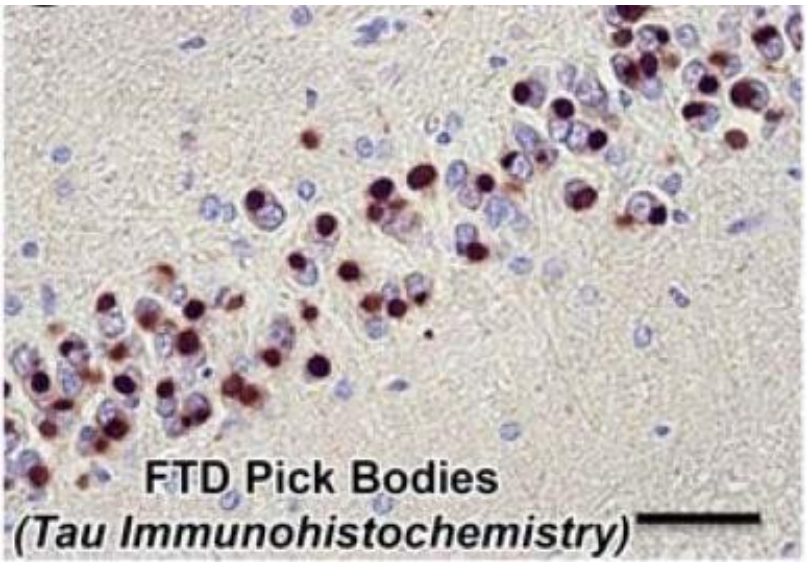


Figure 6; A tau stained immunohistochemistry image to show the tau presence within FTD pick bodies in the neocortex at 50μm (image taken from; Niedowicz, Nelson, Murphy, 2011).

Frontotemporal Lobar Degeneration (FTLD) is the term used for pathological conditions that cause degeneration of frontal and temporal lobes. FTD is a heterogeneous spectrum disorder with very distinct clinical phenotypes associated with multiple neuropathologic substrates (Onley, 2017). The neuropathological classification of FTLDs is split into five major subgroups as shown in figure 8, three of which are characterized by specific proteinaceous inclusions: tau in FTLD-tau, 43 kDa transactive response DNA-binding protein (TDP-43) in FTLD-TDP and fused in sarcoma (FUS) in FTLD-FUS. TDP-43 and FUS abnormal protein

inclusions are shown in figure 7, these abnormal proteins are more associated with FTD subtypes; svFTD & PPA-FTD. The protein inclusions of the fourth group has not yet been identified so is currently classified as FTLD-UPS with charged multivesicular body protein 2B (CHMP2B) mutation. The fifth subtype doesn't contain any protein inclusions and are classified as FTLD-ni (Lashey et al, 2015). The classification of FTLD subgroups is shown in figure 8. Being able to detect these different proteins and thus differentiate between the different forms of FTD would be beneficial in that it would improve early diagnosis and disease management.

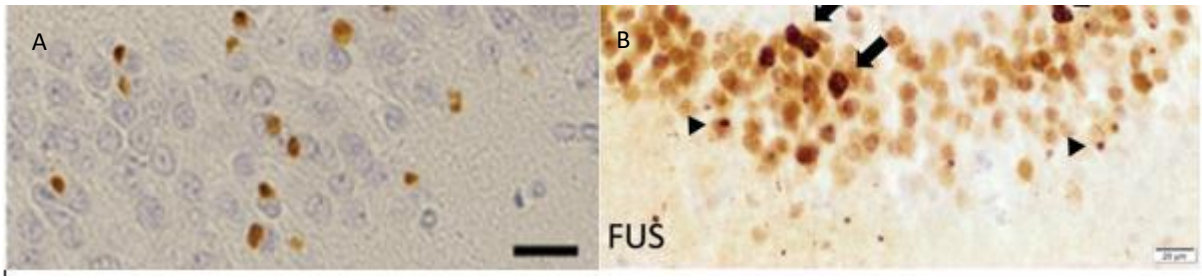


Figure 7; An immunohistochemistry image to show the morphology of the neuronal cytoplasmic inclusions in TDP-43 and FUS abnormal accumulation in FTD. Image A - TDP-43 neuronal cytoplasmic inclusions in the entorhinal cortex, stained with phosphorylated TDP-43-specific antibody (pS409/410) with a bar scale of 20 µm (Image taken from Kawakami et al, 2019). Image B - FUS neuronal cytoplasmic inclusions in the hippocampus stained with FUS antibody (Sigma-Aldrich, HPA008784 with a bar scale of 20µm (image taken from Gowell et al, 2020).

Hyperphosphorylated tau could potentially be the common pathological protein in both AD & FTD (in some subtypes, at least) therefore, to be able to create a specific biomarker for diagnostic tests for different tau forms or tau targeting therapeutic methods, the biochemical structure and function of tau needs to be fully understood. The pathological hallmarks in bvFTD are hyper-phosphorylated and ubiquitinated TDP-43 deposits in up to 50% of cases, 40% of cases are associated with FTLD-tau and the remaining 10% of cases are FTLD-FUS (Bahia, Takada & Deramecourt, 2013).

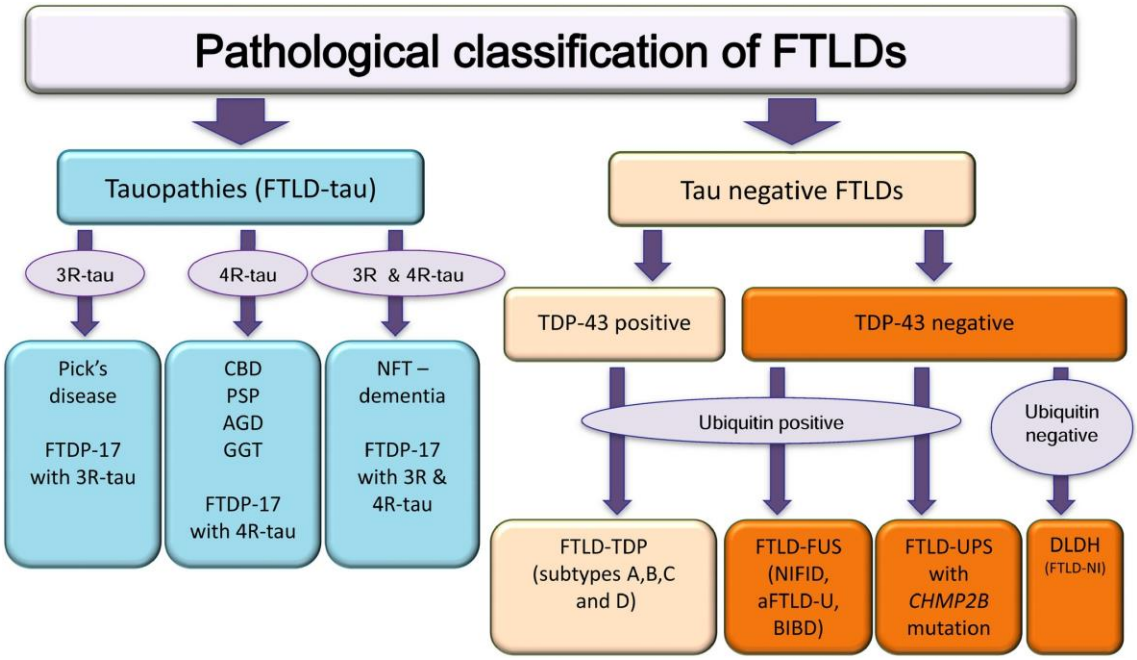


Figure 8: A flow chart to understand the pathological classification of the different subtypes of FTLD. The classification flow chart is based on the different proteins associated with the different subtypes of FTLD (Lashey, Rohrer, Mead & Revesz, 2015).

FTD is characterised by the atrophy which occurs in the frontal and the temporal lobes within the first stages of disease whereas in AD the main regions affected in the first stages of disease are the parietal lobe, the temporal lobe, including the hippocampus and entorhinal cortex (DeTure & Dickson, 2019). It remains unclear why the 'starting' location for early disease changes is different, but halting cell to cell progression could help contain the damage to a specific region.

Eventually AD and FTD patients may experience some of the same symptoms due to the temporal lobe being affected in both types of dementia in earlier stages of disease however, in the later stages of disease all brain regions are affected causing AD and FTD to end in a similar way experiencing similar symptoms in the final weeks of disease (Mohandas & Rajmohan, 2009). It is not clear on why the diseases initiates within different brain regions however, evidence has now show that sEVs are involved within the spread of disease by prion-like spread. Investigating the spread hypothesis in disease could allow therapeutic agents to target sEVs help slow down disease progression or even stop the spread of disease. Limiting the spread of disease would have less impact on physiological functions and cognitive decline. Being able to successfully isolate and study sEVs cargoes from living

patients could also provide a novel biomarker source to help with early disease detection and differentiation between the different drivers of disease.

1.6.1 Abnormal protein accumulation risk factors

AD is thought to have many associated risk factors and overlapping pathophysiologic mechanisms however, oxidative stress appears to be one of the major risks associated with disease development (Huang, Zhang & Chen, 2016). Oxidative stress is one of the factors known to increase abnormal protein accumulation increasing the risk of neurodegenerative diseases (Christen, 2000). Oxidative stress is a process that increases as the brain ages and is caused by the imbalance of oxidants and antioxidants causing excess reactive oxygen species (ROS) (Huang, Zhang & Chen, 2016). When the equilibrium of antioxidants and oxidants is disrupted, the imbalance causes an increase in oxidative stress resulting in apoptosis, damaged cells or even cell death (Hoeberichts & Woltering, 2003). However, under physiological conditions there is a balance of ROS formation and antioxidants. It is only when the balance is disrupted does it result in pathological situations leading to cell death (Huang, Zhang & Chen, 2016). ROS are known to react with lipids, proteins and other molecules and could potentially alter their structure and function resulting in pathological damage (Huang, Zhang & Chen, 2016). Oxidation of the brain can affect DNA, producing DNA-protein cross linking and DNA base pair modification; causing mutations. DNA mutations will affect the end product; the protein, dysfunctional proteins contribute to pathological problems including the neurodegenerative disorders (Cooke, Evans, Dizdaroglu & Lunecl, 2003).

Brain inflammation is another major factor that many researchers are interested in, as this has also been linked to the abnormal accumulation of protein and the neuropathological changes seen in AD (Kinney et al, 2018). Other research studies suggest that brain inflammation has a dual function, as it plays a neuroprotective role but can also be involved in damage during a chronic inflammatory response (Yoon & Tong, 2006). Aβ deposits have been shown to be increased in response to evaluated levels of Interleukin 1(IL-1) in patients with head injury, and this also increases the risk of AD. IL-1 is also linked to the increase in

APP production and Aβ load therefore, elevated levels caused by head injury could result in the overproduction of Aβ, contributing to the increased AD risk (Goldgaber et al 1989). The elevation of IL-1 also increases the production of other cytokines, including interleukin 6 (IL-6). IL-6 activates CDK5 which is known to induce tau protein hyperphosphorylation (Quintanilla et al, 2004). Exacerbating Aβ burden and tau hyperphosphorylation are also known to induce neuroinflammation (Kinney et al, 2018) therefore, neuroinflammation has a role in the pathology of both AD and FTD syndromes (Miller et al, 2013). These inflammatory markers could be detectable in both brain and peripheral tissues, but it is unclear if these markers, of their associated genetic regulators, are detectable in sEVs.

1.6.2 Genetic risk factors

Despite both AD and FTD being characterized by abnormal protein accumulations, they are associated with different genetic mutations. Dementia is a multifactorial disease with numerous genetic and environmental risk factors. FTD is a neurodegenerative disease with a high heritable risk, at least 40% of FTD cases is familiar, with between 10% - 30% of FTD familiar cases have family history with the *MAPT* genetic mutation. 48% of diagnosed FTD cases are made up of the bvFTD subtype and 12% of the cases are related to the PPA subtype (Ghosh & Lippa, 2015). There are three main genetic mutations associated with FTD; *MAPT* on chromosome 17, progranulin (*PGRN*) on chromosome 17 and (*C9orf72)* chromosome 9 open reading frame 72 being the most common genetic contributor worldwide (Greaves & Rohrer, 2019 & Ghosh & Lippa 2015). The genetic mutations *GRN* and *C9orf72* have an increased risk with age unlike *MAPT* which is fully penetrant in most cases. The genetic mutations of FTD are predominately associated with the different subtypes of FTD; *C9orf72* is the genetic mutation mostly associated with bvFTD whilst *GRN* mutation is more likely to result in PPA. The *GRN* mutation results in CBS which may occur alone or with PPA. The presence of *GRN* mutation in FTD-ALS is very rare. *MAPT* mutation is mostly associated with SD and bvFTD and shows no forms or PPA subtype, CBS can be a result of *MAPT* but is extremely rare PSP may occur but although never in FTD_ALS (Greaves &Rohrer, 2019). Over the more recent years, there has been other genetic mutations predominately related to FTD but are less common; FUSopathies (mutations in FUS) and

proteinopathies (mutations in *CHMP2B*) (Greaves &Rohrer, 2019). *C9orf72* is the most common mutation in FTD, ALS and FTD/ALS (Smeyers, Banchi, Latouche, 2021) and even after a decade of research, the loss-of function and the pathogenesis of *C9orf72* is still not fully understood. However, it is known that carriers of *C9orf72* present TDP-43 inclusions within the neurons and oligodendroglia seen within the frontal and temporal cortex, hippocampus and within the pyramidal motor system but TDP-43 negative neuronal cytoplasmic and intranuclear inclusions (Mackenzie, Frick & Neumann, 2014).

The most common genetic mutations of AD are Presenilin-1 (*PSEN1)*, Presenilin-2 (*PSEN2*) and amyloid-beta precursor protein (*APP*) only 2% of AD cases are inherited, 98% of cases are sporadic (Fedeli, 2019) unlike AD, FTD is more a genetic related neurodegenerative disease whereas AD is more sporadic meaning there are more environmental risk factors associated.

Apolipoprotein E (*ApoE*) is a multifactorial protein that plays important roles within lipid metabolism, neurobiology and neurodegenerative diseases. There are 3 different isoforms, each with different effects on lipids and neuronal homeostasis (Huang & Mahley, 2014). *ApoE* is the most common genetic risk factor with allele ε4 being the AD risk factor whereas, ε2 is known to act as a protective mechanism (Huang & Mahley, 2014). *ApoE* plays a key role in transporting lipids among to different cells and specific tissues as well as, regulating the clearance of lipoproteins from the plasma via specific cell surface receptors including; Heparan sulfate protegeoglycans (HSPGs) (Mahley & Huang, 2012). ApoE synthesis is induced as part of the repair response however, when *ApoE* is expressed within neurons it results in a generation of neurotoxic fragments which can contribute to pathological mitochondrial dysfunction and cytoskeletal alterations (Mahley & Huang, 2012).

The physiological functions of *APP* include the involvement in; cell proliferation, cell differentiation and maturation of neural stem cells (Coronel et al, 2019). *APP* is a transmembrane protein that gives rise to Aβ peptides after undergoing proteolysis and is thought to be a multimodal protein due to being involved in the brain of an embryo as well as present in adults (Coronel et al, 2019).

One of the genetic risk factors associated with FTD is the mutation of the *MAPT* gene which plays a critical role in enhancing the microtubule assembly and stability which is important for neurite growth, cell stability, intracellular transport as well as maintaining the axonal health (Kadavath et al, 2015). Mutations in tau are known to cause a disruption in cellular interaction resulting in the destabilisation of microtubules which increases the risk of unbound but also free-floating tau which is thought to promote the aggregation of tau increasing the risk of AD and FTD (Kadavath et al, 2015).

The other two genetic mutations associated with FTD are *GRN* & *C9orf72.* Both og theses genetic mutations play an important role within the autophagy pathway and mutations within both pathways cause the autophagy process to be ineffective (Smith et al, 2012 & Amick, Roczniak-Ferguson & Ferguson, 2016). *GRN* gene also downregulates microglial neuroinflammation as well as influencing both lysosomal activity and autophagy. The mutation of *GRN* results in a lysosomal storage disorder which causes defects in autophagy contributing to FTD-GRN pathology (Smith et al, 2012). The *C9orf72* gene is known to directly affect lysosomal function which affects the function of endolysosomal trafficking and autophagy resulting in abnormal protein accumulation (Amick, Roczniak-Ferguson & Ferguson, 2016).

Presenilin's (PS) are a family of transmembrane proteins located in different cellular structures such as endosomes, lysosomes, nuclear envelope, mitochondria, the trans-Golgi network, and the endoplasmic reticulum (Escamilla-Ayala et al., 2020) which are important cellular structures in relation to autophagy therefore, mutations within *PSEN* genes are known to impair the autophagy pathway contributing to neurodegenerative diseases (Escamilla-Ayala et al, 2020 & Fedeli et al, 2019). *PSEN* genes are well-known for its role within the γ-secretase complex which is ultimate enzyme that is responsible for Aβ peptides and Aβ accumulation formation the most famous pathological hallmark for AD (Fedeli et al, 2019 & Escamilla-Ayala et al, 2020). In addition, *PSEN2* is responsible for the modulation of intracellular Ca^{2+} homeostasis. The mutated *PSEN2* gene is known to impair the autophagy pathway by causing a block in the degradative flux at the level of the autophagosome-lysosome fusion step enhancing abnormal protein accumulation and aggregation (Fedeli et

al, 2019). The role of autophagy and its importance within neurodegenerative diseases will be discussed in chapter 'Autophagy and Dementia'.

The genetic and pathological heterogeneity associated with the different subtypes of dementia has made it challenging to correctly therapeutically target the underlying disease drivers and so identification of prodromal biomarkers could shift the field of dementia research and patient care.

1.6.3 Environmental risk factors

There are many environmental risk factors associated with dementia and these could influence physiology at a systemic and epigenetic level. At least a third of AD cases are thought to be influenced by health and lifestyle choices, such as smoking, being overweight, diabetes and depression (Livingston et al 2017). As dementia related diseases are associated with many environmental risk factors as well as genetic mutations it is incredibly hard to understand and diagnosis correctly due there being multiple disease drivers (Killin et al, 2016). Environmental/ medical factors that are mainly associated with FTD include hypertension, hyperlipemia, diabetes, heart disease, smoking, exposure to chemicals and pesticides (Rosso et al, 2003). Unlike AD, traumatic brain injury does not currently appear to increase the risk of FTD (Kalkonde et al, 2012). The accumulative impact of environmental and genetic risk factors is a difficult thing to study, but low cost and minimally invasive methods to allow this to happen over the course of disease would be invaluable to the field.

1.7 Treatments available

The current treatment methods available for AD & FTD include pharmaceutical treatments and lifestyle changes to address symptoms, but there remain no approved disease modifying therapies in the UK to slow or stop the progression of AD (Zhang et al 2021). The main treatment options available to dementia patients are prescribed medications that help to manage the behavioural symptoms for example, sleeplessness, depression, agitation and restlessness. The prescribed medications include sleep aids, anti-anxiety drugs,

antipsychotics and anti-convulsants (Yohanna & Cifu, 2017). The other treatment method available for dementia patients are acetylcholinesterase inhibitors; donepezil, rivastigmine and galantamine that are recommended therapies for patients with mild, moderate and severe AD (Birks, 2006). Acetylcholinesterase inhibitors despite having different pharmacological properties work by inhibiting the breakdown of acetylcholine, which is an important neurotransmitter associated with memory and thinking, by blocking the enzyme acetylcholinesterase (Birks, 2006). However, these drugs can only enhance neurotransmission while cells are alive. Amyloid targeting drugs have had some recent success in clinical trials however, they are not yet approved for use in the UK. Aducanumab is a monoclonal antibody that targets amyloid plaques (Sevigny et al, 2016) and more recently, Lecanemab. Lecanemab is a disease modifying drug used target β-Amyloid plaques and clear the β-Amyloid from the brain reducing the amount of pathology present to slow down cognitive impairment (van Dyck et al, 2023). Targeting the abnormal proteins would be a possible therapeutic method to cure dementia, the removal of Aβ plaques and NFTs will stop the abnormal accumulation of pathogenic proteins & stop pathogenic spread to other brain regions causing neuronal death leading onto atrophy of brain region causing cognitive impairment therefore, the earlier the Aβ plaques and NFTs are detected the less damage is done (Braak & Del Tredici, 2013). According to studies, the Aβ plaques start to accumulate decades before any symptoms manifest therefore, a biomarker which could possibly determine what type of abnormal protein was accumulating so people are able to receive a correct diagnosis and get the right treatment earlier on in disease (Bateman et al 2012). Many current studies are investigating ways of eliminating abnormal protein accumulation and infact, the body has its own system to do exactly that: autophagy.

1.8 Autophagy

Autophagy is a self-degradative process that the human body relies on to clear out aggregated/misfolded proteins, remove any dead organelles as well as eliminating intracellular pathogens. This system removes long-lived proteins, aggregated proteins and damaged organelles from the body (Glick et al2010). Understanding why this system (or

group of systems) does not target the abnormal proteins associated with AD and FTD could help identify approaches to restore the normal clearance function.

1.8.1 Types of Autophagy

There are 3 different forms of autophagy; microauthophagy, macroautophagy & chaperone-mediated autophagy (CMA) (Mizushima, 2007). As seen in figure 9 the different types of autophagy have the same end result (lysosomal degradation to eliminate cellular waste). The autophagy pathways can be either selective or non-selective in the removal of waste products and is seen to be a survival system despite resulting in cell death (Glick, Barth & Macleod, 2010).

CMA was the first process of autophagy to be discovered, this type of autophagy is selective and degrades waste products within lysosomes after they have been directed there by a chaperone protein (Kaushik & Cuervo, 2018). CMA is a lysosomal pathway responsible for at least 30% of cytosolic protein degradation (Dice, 2007). CMA utilizes chaperone proteins such as; heat shock cognate protein 70 (HSC70), heat shock protein 90 (HSP90) and heat shock protein 40 (HSP40) to recognise proteins with a KFERQ motif and it does not utilize any membrane structure to engulf cargo. Once the protein cargo has been recognised by the molecular chaperones in within the cytosol, the protein substrates are unfolded before being sent across the lysosomal membrane for degradation (Cuervo & Wong, 2014). Prior to the translocation of substrate, lysosome-associated membrane protein 2A – LAMP2A, is required to pull the unfolded substrate across the lysosomal membrane (Dice, 2007). CMA and microautophagy are responsible for clearing out soluble neurotoxic tau whereas intracellular insoluble tau is degraded by macroautophagy (Wang et al, 2009 & Caballero et al, 2018). CMA is involved in a number of physiological functions as well as autophagy such as; DNA repair, cell reprogramming, responding to cellular stress and glucose and lipid metabolism. The key role of CMA is protein quality control and the failure of CMA can result in neurodegenerative diseases (Kaushik & Cuervo, 2018). CMA is thought to be involved in the degradation of TDP-43 protein inclusions, a pathological protein responsible for FTD (Budini et al, 2015).

As seen in figure 9, micro-autophagy is the shortest autophagy pathway and was considered to be a non-selective pathway, which internalises soluble cytosolic proteins through invaginations of lysosomal membrane itself and can engulf large amounts of waste (Sahu et al, 2011). However, a selective form of micro-autophagy has been discovered with the recognition of *KFERQ* motifs and with the help from HSC70, that delivers cytosolic proteins to the late endosome. This selective type of micro-autophagy is known as endosomal micro-autophagy (e-MI) (Sahu et al, 2011). The main physiological functions of microautophagy include; membrane homeostasis, the maintenance of organellar size and cell survival (Li, Li & Bao, 2012).

Macroautophagy is the most complex form of autophagy and is able to degrade proteins via a non-selective or a selective autophagy process (Johansen & Lamark, 2020). Macroautophagy is finely tuned to maintain cell and tissue homeostasis. Autophagy is induced if cells experience stressful situations such as starvation, hypoxia and infection (Kroemer, Mariño, & Levine, 2010). It is important that autophagy is regulated, to reduce the risk of too much self-degradation happening since this leads to cell death and would lead on to an impairment in cognitive decline (Klionsky & Codogno, 2013). The selective macroautophagy process works by creating a double-membrane structure called autophagosome forms around protein aggregates, damaged organelles, and/or microbe prior to their engulfment (Johansen & Lamark, 2020). The autophagosome fuses with a lysosome to create an autolysosome where protein degradation happens (Glick, Barth & Macleod, 2010). Macroautophagy is triggered with the recognition of macroautophagy substrates by selective autophagy receptors (SAR), such as p62/SQSTM1 (sequestosome-1). p62 binds to the ATG8 proteins within the inner membrane of the autophagosome, through LC3-interacting region (LIR) motifs (Johansen & Lamark, 2020). Macroautophagy is able to degrade intracellular insoluble tau (Kruger et al, 2012) and is more recently believed to be involved in the clearance of Aβ and FUS inclusions (Tarasoff-Conway et al, 2015 & Ryu et al, 2014).

p62 is mainly known for promoting autophagy degradation as well as being a multifunctional protein located throughout the cell with many other physiological functions

(Lui et al, 2016). p62 is a protein involved in signal transduction pathways and proteasomal degradation of ubiquitinated proteins but despite, being involved in physiological functions to maintain a cells function, altered p62 levels impact cell survival and can lead onto disease (Lui et al, 2016). The role of p62 within neurodegenerative diseases will be discussed further in chapter "p62 / Sequestsome 1 (SQSTM1) autophagy marker".

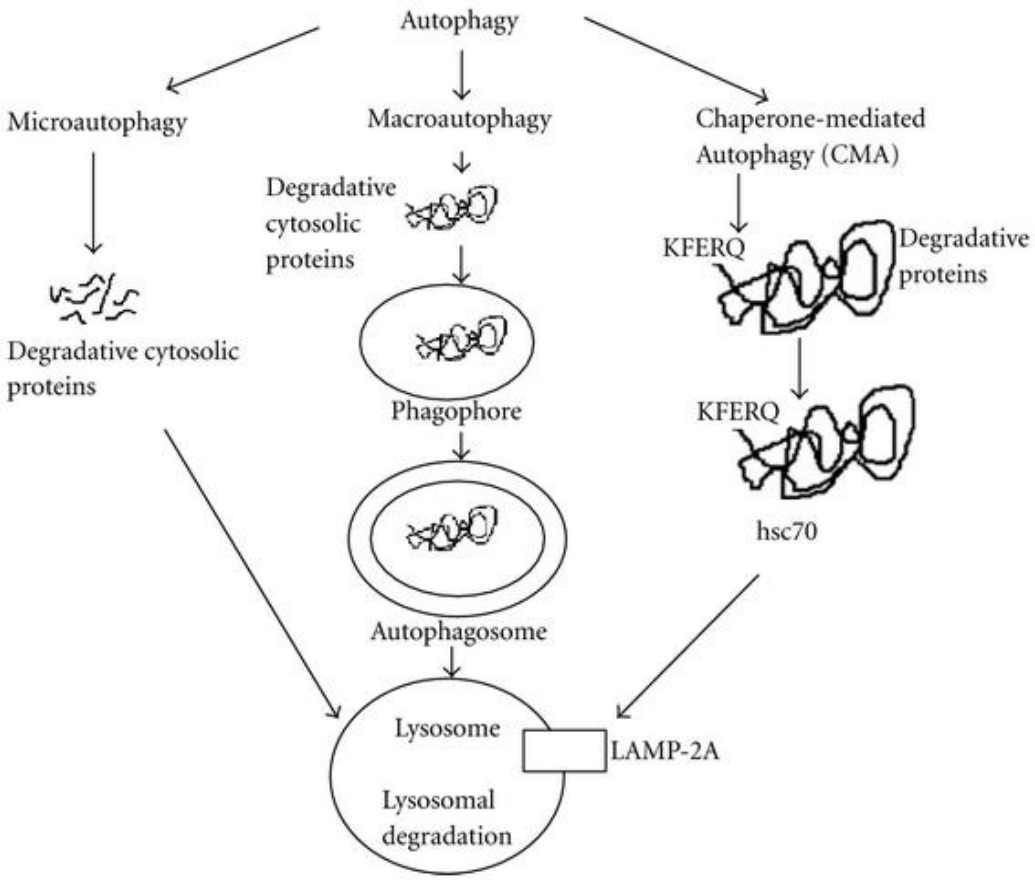


Figure 9; The 3 different autophagy type systems used in clearing the degradation of targeted proteins and damaged organelles as well as fighting intracellular pathogens (Badadani, 2012).

1.8.2 Autophagy and Dementia

The autophagy-lysosomal pathway is an important pathway for removing insoluble proteins within neurons and glial cells, the functioning of this pathway is critical for the degradation of abnormal aggregations (Piras et al, 2016). Despite, the autophagy system being a survival

mechanism to remove abnormal protein (Glick, Barth & Macleod, 2010). Impairments within the lysosomal degradation pathway is thought to contribute to accumulation of abnormal proteins resulting in neurodegenerative diseases such as; AD, PSP and CBD (Piras et al, 2016). Wang & Mandelkow 2012, also suggests that endolysosomal failure from lysosomal storage defect, may be potentially inducing tau aggregation and tangle build-up in the brain. The degradation failure of pathological proteins via macroautophagy has been reported in a number of studies; the lack of removal of these pathological protein increases the risk of abnormal protein accumulation resulting in neurodegenerative diseases (Wang & Mandelkow, 2012). It has been observed in post-mortem brains of familial AD patients that hyperphosphorylated tau co-localizes with LC3-positive vesicles and p62 receptor protein (Piras et al, 2016). The presence of p62 and LC3 will induce the macroautophagy process to try and remove the hyperphosphorylated tau (Johansen & Lamark, 2020) which shows the important of the macroautophagy system and the importance of the system functioning correctly in neurodegenerative diseases. A disruption within the balance of protein production and degradation can contribute to the accumulation of abnormal protein resulting in, characteristics of neurodegenerative disorders relating to amyloid-β (Aβ) and hyperphosphorylated tau in FTD and AD (Caccamo et al, 2017).

A high concentration of HSC70 activity which correlates with reduction of tau in cells and animal models (O'Leary III et al, 2010). This evidence shows a positive relation between tau protein and HSP70 and the importance of autophagy markers as well as the importance of a fully functioning autophagy system. A deficiency of Beclin1 results in an increase of *APP* and Aβ whereas, an over expressed beclin1 shows to reduce the accumulation of Aβ (Pickford et al, 2008). The genetic reduction of beclin1 is known to not only increase intraneuronal Aβ but extracellular Aβ disposition as well as neurodegeneration (Pickford et al, 2008). Tung et al, found that the genetic mutation PS1 associated with AD exhibit reduced levels of p62, p62 is an important SAR which triggers autophagy therefore, the process of protein degradation could be delayed or may not happen at all increasing the risk of tau accumulation if the autophagy system was not in working order (Wang et al, 2009). The restoration of autophagic pathways could be a potential method to treat neurodegenerative diseases, if we are able to understand which parts of these pathways are not effective. Biomarkers that expose potential autophagy deficits have not yet been identified and it is

not clear if autophagy pathway markers can be identified within sEVs. Being able to identify early on which autophagy pathway is impaired would help with therapeutic targeting to prevent the damaging effects of abnormal protein accumulation in AD and FTD.

1.8.3 p62 / Sequestsome 1 (SQSTM1) autophagy marker

p62 is an important multifunctional protein involved in the process of removing short lived, misfolded and damaged proteins (due to abnormal protein modifications and mutations), via autophagy and via the proteasome. p62 is presence during the process of aggrephagy which is the process of intracellular protein aggregate formation (Stolz, Ernst & Dikic, 2014) and so p62 co-localises with numerous proteinaceous aggregates, including those associated with AD and FTD. Protein degradation is important to maintain protein homeostasis, cell cycle control, inflammation and apoptosis within cells (Lui et al, 2016 & Caccamo et al, 2017). p62 is a classic receptor that contains a ubiquitin-binding associated domain (UBA) and a LIR. p62 binds to polyubiquitinated proteins including tau, through the UBA domain and targets for proteasome degradation. Through its LIR domain, p62 binds to LC3 to facilitate selective macroautophagy to start process of protein degradation (Pankiv et al, 2007). Mutations within the p62 gene are known to be associated with neurodegenerative disease such as ALS and FTD (Fecto et al, 2011). p62 plays an important role in neurodegenerative diseases such as AD & FTD in both in tau aggregation and degradation. Strong evidence highlights the importance of p62 using knockout age dependent mice which demonstrated accumulation of NFTs and synaptic deficits (Caccamo et al, 2017). In AD, the autophagy substrate p62 also attempts to target the NFTs via delivering the tau protein to the proteasome for degradation. However, this process can be complicated due to p62 being hidden within NFTs, which may limit its cellular function by creating reduced levels of available p62. Studies suggest that increasing the p62 levels could potentially be a therapeutic method for neurodegenerative diseases, although the increased levels of p62 does not change proteasome activity but enhances targeting (Lui et al, 2016 & Caccamo et al, 2017). Studying P62 in human brain tissue gives a snapshot of both protein aggregate formation as well as insight to protein degradation activity. Whilst it

is known that sEVs have been implicated in the pathological spread of Aβ42 oligomers and mutant tau within the different brain regions (Schneider et al 2018) it is unclear if protein degradation associated proteins such as P62 can be detected within sEVs and so this is an important and novel area of research.

1.9 Extracellular vesicles (EVs)

EVs can be characterised and split up into two main subtypes with regards to their cargoes and their biogenesis pathway; ectosomes and sEVs (Théry et al, 2018). Ectosomes are roughly between the size of 100-1000nm and are generated by cytoplasmic membrane budding. However, sEVs are between the size of 30-200nm and created by the fusion of the of the large multivesicular bodies (MVBs) also known as intraluminal vesicles (ILVs) which form within the plasma membrane and are then released into the extracellular space as sEVs (Théry et al, 2018).

The functions of sEVs in physiological and pathological states will discussed further in the following chapter.

1.9.1 Small Extracellular vesicles (sEVs)

In the first instance, sEVs were thought to be part of the waste disposal mechanism of cells however, in the most recent decade sEVs are the forefront of research to investigate their different biological and pathological functions given their clear role in cell to cell communication (Negahdaripour et al, 2021). sEVs are lipid bilayer-enclosed extracellular vesicles between 30-200nm in size that contain various molecular constituents including proteins, lipids, RNA and DNA (Negahdaripouret al, 2021). They are involved in the intracellular communications (Liu et al, 2019) and influence so many other physiological functions (Woith, Fuhrmann & Melzig, 2019). sEVs are not able to replicate but are released from almost all living cells into the extracellular environment (Woith, Fuhrmann & Melzig, 2019) and can influence immunoregulation, central nervous system development, regeneration of tissues, inflammation, angiogenesis, coagulation and apoptosis (Negahdaripour et al, 2021). They have also been implicated in a number of diseases such

as, stroke, AD, Parkinson disease (PD) and prion disease, being reported to have both positive and negative effects including the transfer of misfolded proteins (Liu et al, 2019). sEVs contain bioactive contents which can be investigated to understand the intracellular environment in any given physiological state as well as in disease states. sEV contents are known to change with disease occurrence making sEVs an intriguing hope for an early detection biomarker in neurodegenerative diseases. sEVs are known to be able to penetrate the BBB, making sEVs an ideal therapeutic for brain associated diseases (Liu et al, 2019) and the ability to give insight on the neurodegenerative environment. The relationship between sEVs and the BBB will be discussed further in the following chapter.

1.9.2 Small Extracellular vesicles and the blood brain barrier

The blood brain barrier (BBB) maintains homeostasis of the central nervous system (CNS) as well as having a selective protective mechanism to protect neural tissue from toxins (Daneman & Prat, 2015). As shown in figure 10, the tight junctions between the endothelial cells act as a physical barrier only allowing small molecules to pass through (Saint-Pol et al, 2020). sEV are small particles that are able to cross the blood brain barrier for treatment purposes or to shed light on the neurological cellular environment and this, is what makes sEVs so interesting in neurodegenerative diseases (Ramos-Zaldívar et al, 2022 & Zhou,Smith, & Liu, 2021). Due to their potential purposes in diagnostic and therapeutic applications, in the recent decade research within the sEV field has increased significantly in the neurodegenerative, cancer and immunology field (Samantha et al, 2018). The full potential uses of sEVs within the therapeutic and diagnostic field are shown in figure 11.

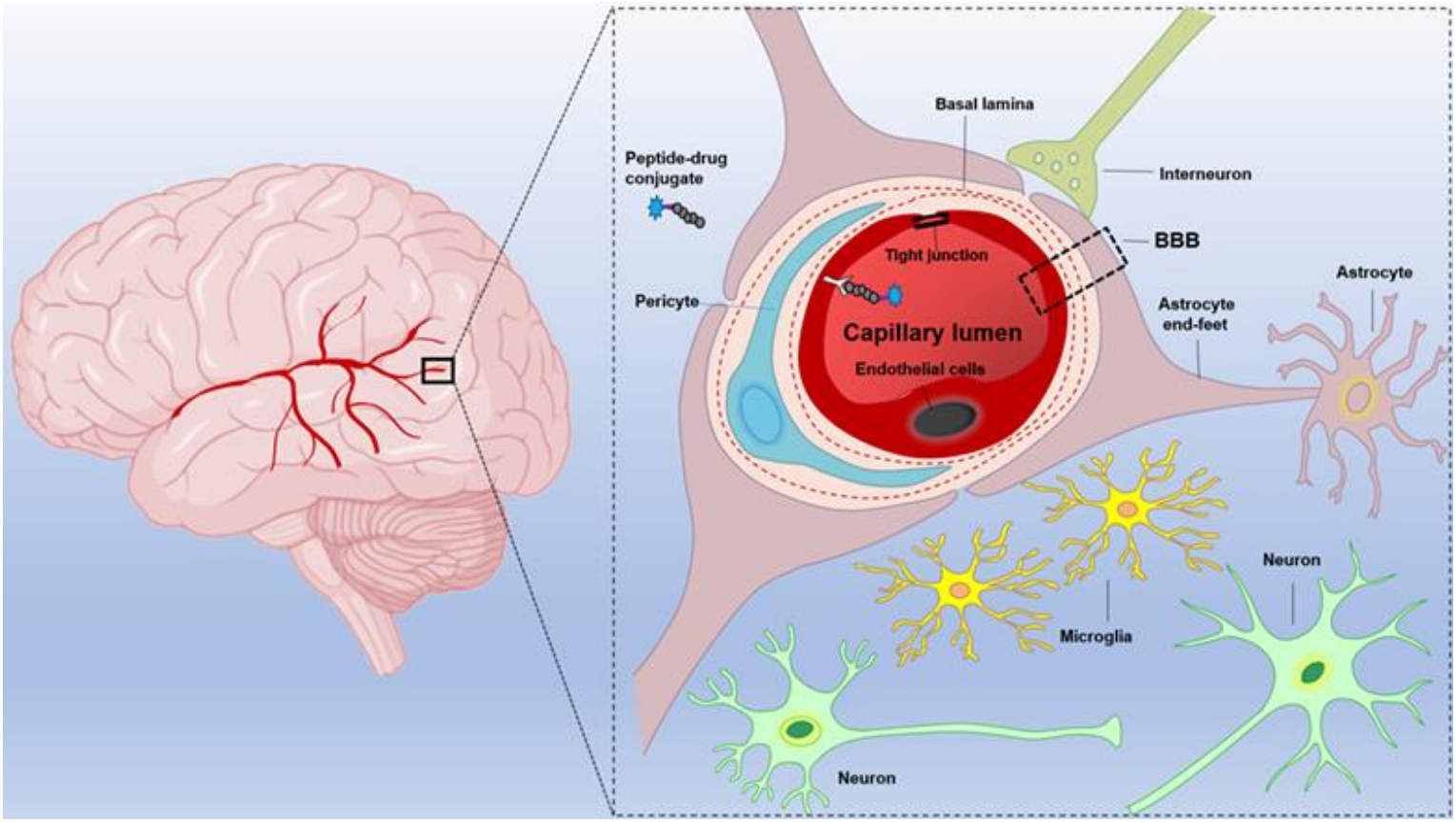


Figure 10; The blood brain barrier is the most complex mechanism within the human body and although the main function is to act as a protective mechanism it makes it incredibly difficult to treat brain diseases such as neurodegenerative diseases (Zhou,Smith, & Liu, 2021).

1.9.2 sEVs as a biomarker and therapeutic

sEVs are involved in the intercellular communication, are detectable in tissues, circulation, and body fluids and with their complex cargo reflecting the (patho)physiologic status of the cells from which they originate from. sEV contents therefore have the potential to serve as biomarkers or as a vehicle for the delivery of therapeutic agents. Due to these properties and with the potential sEVs have, the interest has grown exponentially over the past decade (Ciferri, Quarto & Tasso, 2021). sEVs are known to remain stable whilst circulating within body and are also found in all body fluids, some which are considered to be none / less invasive such as; urine and blood (Boukouris & Mathivanan, 2015). sEVs contain a complex cargo which could possibly be used as an early detection method in diseases as well as being

compared to healthy donors. Circulating sEVs derived from cancer patients had a positive correlation with survival, patients with higher levels of circulating sEVs had a shorter survival compared to those patients who had low levels of sEVs (Silva et al, 2012). sEVs are known to increase as the tumor microenvironment matures therefore, sEVs are known to play a part in tumor progression. There are 3 ways to inhibit sEV production to decrease the tumor progression rate; inhibition of sEV secretion, elimination of circulating sEVs and disruption of sEV absorption (Naito et al, 2017). sEVs are an endogenous delivery system which delivers their contents to recipient cells therefore, therapeutic applications of sEVs as a drug delivery systems of various drugs are expected to be investigated to treat disease (Takakura, Matsumoto & Takahashi, 2020). As shown in figure 11, there are a number of potential uses for sEVs within the medical field, from diagnosis, to acting a drug delivery system to being involved in vaccines and treatment methods sEV are the hope in many diseases.

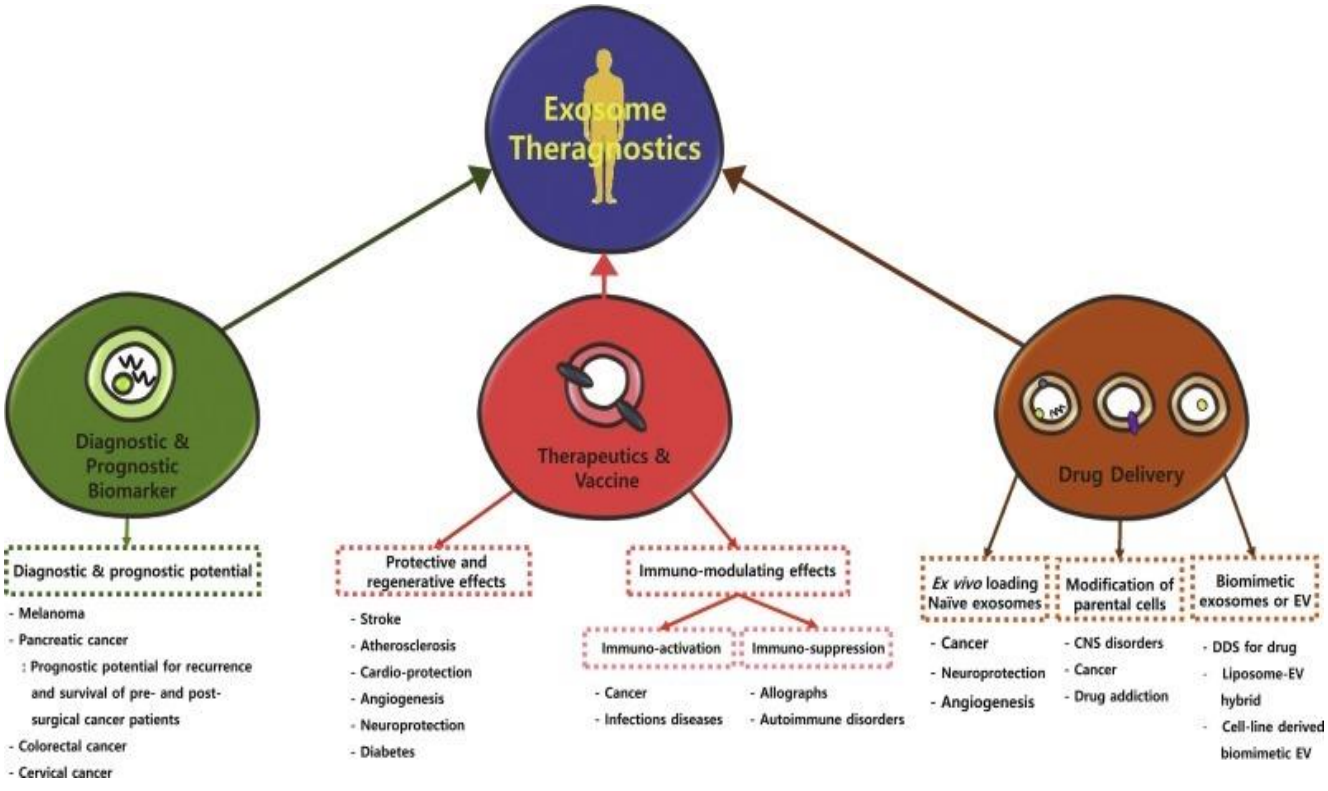


Figure 11; Small extracellular vesicles show many potential uses as a multifunctional theragnostic in different diseases.

1.10 miRNAs biogenesis and function

One of the many cargoes of sEV's are miRNAs, which functions to control gene expression and thus could contribute to dysregulation of key genes associated with dementia (Hu, Drescher & Chen, 2012). miRNAs are a small group of single-stranded non-coding RNA with a length of ~25 nucleotides long and the biogenesis of miRNAs is a complex process consisting of many steps. The process starts in the nucleus and is completed in the cytoplasm, this is where the mature miRNA pairs with the target messenger. The biogenesis of miRNA is classified into either canonical or non-canonical pathway (see figure 12).

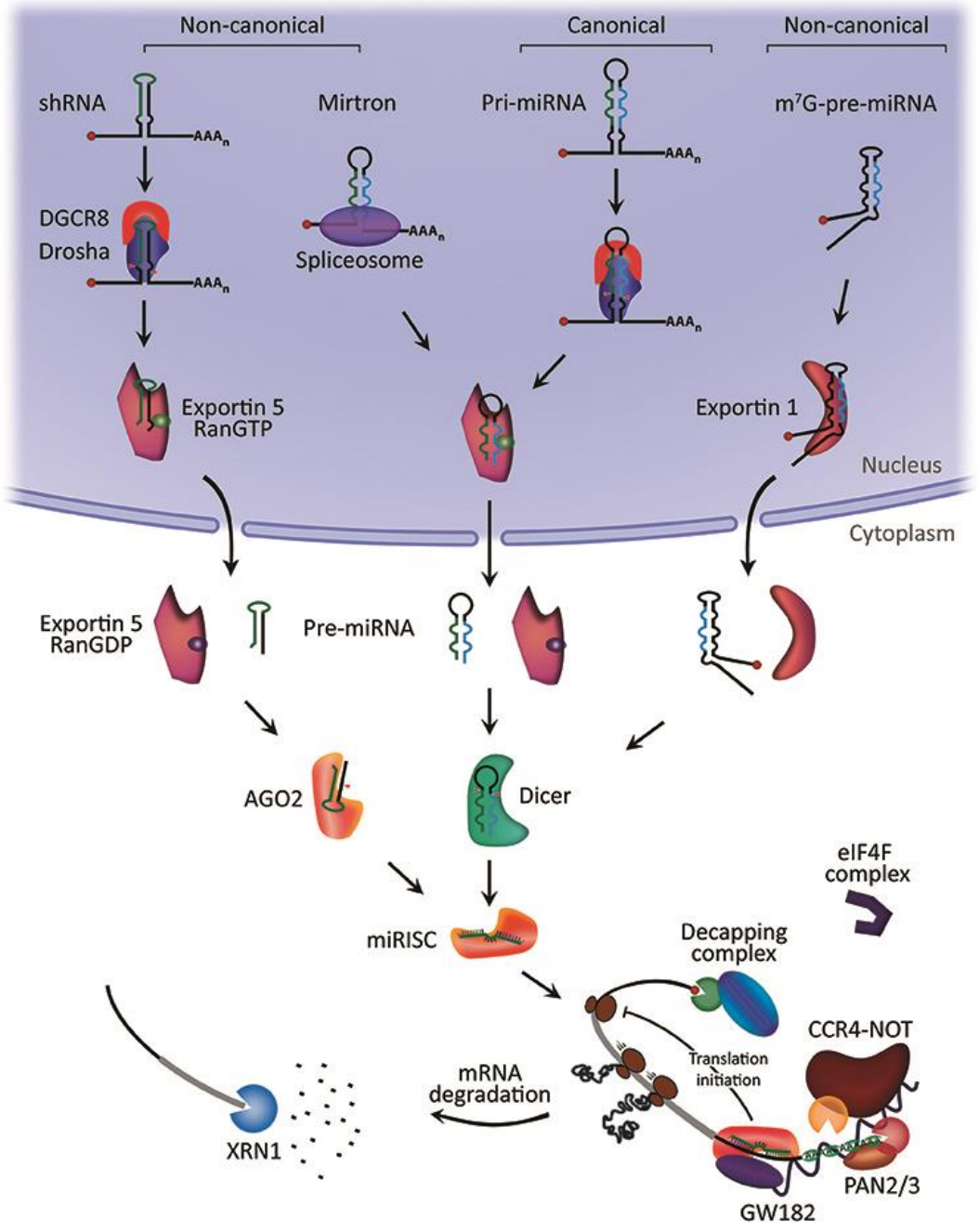


Figure 12; A figure to demonstrate the biogenesis of miRNAs through the canonical and the non-canonical pathway. Edited by Hayder et al 2004.

The canonical pathway is the most common pathway in which miRNAs are processed. The pri-miRNAs are transcribed from their genes and processed into pre-miRNAs by the microprocessor complex that consists of an RNA binding protein and a ribonuclease enzyme. The RNA binding protein is DiGeorge Syndrome Critical Region 8 (DGCR8) and the ribonuclease enzyme is Drosha (Denli et al, 2004). The RNA binding protein DGCR8 recognises an N6-methyladenylated GGAC within the pri-miRNA, whilst the ribonuclease enzyme Drosha cleves the pir-miRNA duplex at the base of the hairpin structure of pri-miRNA which formats the 2 nt 3' overhang on the pre-miRNA (Han et al, 2004). Once the miRNAs have been generated, they are exported out of the cytoplasm and processed by the RNase III endonuclease dicer (Han et al, 2004). This process removes the terminal loop which results in the mature miRNA (Denli et al, 2004).

The non-canonical pathway still uses a combination of proteins which were used within the canonical pathway such as; Drosha, Dicer, exportin 5, and AGO2. The non-canonical pathway is grouped into 2 subgroups; the Drosha/DGCR8-independent pathway or the Dicer-independent pathways (Ruby et al, 2007). The pre-miRNAs which are produced via the Drosha/DGCR8-independent pathway resemble dicer substrates, which are directly exported out of the nucleus and into the cytoplasm via exportin 1, without the need of Drosha cleavage (Babiarz et al, 2008). However, the Dicer-independent miRNAs are processed via Drosha from short hairpin RNA transcripts. This type of pre-miRNAs needs AGO2 to complete the maturation process within the cytoplasm due to the length being insufficient to be dicer-substrates (Berezikov et al, 2007). This assists the loading of the pre-miRNA into AGO2 and then the AGO2-dependent slicing of the 3p strand. The 3'-5' trimming of the 5p strand completes the miRNA maturation process (Okamura et al, 2007).

miRNAs inhibit the gene expression by binding and to the complementary sequences in the 3' untranslated region (3'UTR) of mRNAs to target them for degradation and prevents the mRNA from being translated (Bartel, 2009). It has been shown that 1 single miRNA can prevent the translation of mRNAs by alteration of the seed sequence which is 2-8 nucleotide from 5' end (Bartel, 2009). The alteration of seed sequence will result in an increased of inhibition of translation or degradation of their targets. If the seed sequence is complementary to the 3 UTR region degradation happens, but if the complementary

process is imperfect the process of translation will be blocked (Manna et al, 2020). miRNAs also take part in other cellular processes; homeostasis, cell proliferation, cell differentiation, cell death (Apoptosis) and responding to stress (Nguyen et al, 2022).

A single miRNA alteration can result in many neurodegenerative diseases such as; AD, PD, ALS and Huntington disease (Nguyen et al, 2022) Despite, miRNAs mostly being inside the cell, they are able to migrate out of the cell and circulate, as they can be found in biofluids such as plasma, serum and blood (Hu, Drescher & Chen, 2012). miRNAs have become a very popular choice of becoming a new therapeutic biomarker through their ability to reflect on cellular status, their ability to be measured in bodily fluids (which are less invasive) and their utility as a biomarker in neurodegenerative diagnosis although it has not yet been fully explored, particularly those derived from sEVs (Manna et al, 2020).

1.10.1 miRNAs and Dementia

70% of detectable miRNAs are expressed in the nervous system. Impaired regulation or dysregulation of miRNAs have been linked to neurodegenerative diseases such as AD, PD, epilepsy, multiple sclerosis (MS) and ALS. Recent studies have monitored the regulation different types of miRNAs in AD, FTD and controlled patients. miRNA-342-3p was dysregulated in AD patients and the expression of miRNA-125a-5p, miRNA-125b-5p and miRNA-342-3p was found to be up-regulated in the brains of patients with AD (Dong et al 2020). The evidence sets promising high expectations for an early detection diagnosis in AD & other neurodegenerative diseases with the use of miRNAs as biomarkers. The studies allow scientists to build a profile of potential miRNAs that are upregulated, dysregulated and downregulated in AD & other neurodegenerative diseases for example Dong et al, 2020, stated the levels of ex-miRNA-632 were significantly decreased in sporadic FTD patients compared to healthy controls & CSF ex-miRNA-204-5p and ex-miRNA-632 were significantly decreased in symptomatic patients (Paul et al 2020). miRNAs are known to play a role in the pathological processes of AD; Aβ accumulation, tau-dependent toxicity, inflammation and neuronal death (Manna et al, 2020). The miRNA profile will allow scientists to build a diagnostic tool based on the miRNA's regulation levels and will allow the diagnosis to be

correct and more in-depth for the patient. Although the use of miRNAs as therapeutics face many challenges, the size of miRNAs is one of characteristic that makes miRNAs attractive, when considering therapeutic options (Paul et al 2020). sEV cargoes contain selectively packed miRNA, sEV transport various molecules to the recipient cells including miRNAs (Hu et al, 2012). It is unclear if sEVs could be contributing to the spread of adversary miRNAs in disease or if miRNA levels could be used diagnostically or to shed light on the underlying disease drivers.

1.10.2 sEV – miRNAs

Nerve cells are able to synthesis and release sEVs which cross the blood brain barrier which can be detected in blood and peripheral fluids. It is well established that miRNAs do regulate AD-associated proteins within the brain and are stable in bodily fluids such as CSF and blood as they transport through to recipient cells within sEVs (Dong et al, 2015). Researchers are investigating the sEV miRNA content from serum samples from AD patients as well as healthy patients (Dong et al, 2015). This process is minimally invasive which makes the process more attractive as a source of biomarkers for AD, in comparison to CSF analysis. The miRNA is encapsulated within the sEVs making them free of RNA contaminants (Manna et al, 2020). Yang el al found that by comparing miRNAs, it is not just an early detection biomarker but can also differentiate between some neurodegenerative diseases. The expression levels of miR-135a, miR-193b and miR-384 from serum derived sEVs were investigated across 4 subgroups including AD, PD, VaD and MCI. The results shown the miR-384 was the best microRNA to be used to differentiate between AD, PD and VaD for an early AD diagnosis (Yang et al, 2018) however, this has not been explored in the different FTD subtypes.

Isolating sEVs miRNA from human brain tissue with and without a diagnosis of AD and FTD could build up a profile of those miRNAs which are upregulated and downregulated in different disease types, though this isolation can only be completed after post-mortem. However, attempting to isolate sEVs from post-mortem derived brain tissue in the first

instance (where neurodegeneration associated changes are likely to be more measurable) and the mapping the disease associated cargo changes onto more peripheral tissues such as blood, urine or skin-derived fibroblasts, we would have a more targeted approach to biomarker identification. In this study, methods for the isolation of sEVs from both human brain tissue and commercially available skin-derived fibroblasts, from Alzheimer's and Control patients were explored. Development of these methods is key to enable further studies investigating sEV cargo variation (namely pathogenic protein, autophagy associated protein and miRNA) between AD and the various FTD subtypes in a variety of central and peripheral tissues.

1.11 The future for dementia

AD and FTD are the most common types of dementia under the age of 65 across the globe. The number of people being diagnosed with FTD & AD are increasing, becoming even more of a burden on the society and economy therefore, there is an urgent clinical need of early detection methods and therapeutic methods. Scientists in the recent decade are completing more research on miRNAs and sEVs, and the development of methods to study sEV cargo is increasingly important.

1.11.1 sEVs and Dementia

sEVs have become a promising biomarker as well as therapeutic agent in neurodegenerative diseases with their potential of initiating and participating in the regulation of neuroinflammation (thought to be an inducer of AD) as well as improving neurogenesis and neurogenic physiological location (Yang et al, 2017). The most common feature of neurodegenerative diseases is the accumulation of abnormal insoluble proteins both intracellular and extracellular also known as proteinopathies (Hartmann et al, 2017). AD is characterised by the aggregation of *MAPT* & Aβ, PD is characterised by the accumulation of alpha-synuclein and ALS is characterised by the accumulation of TDP-43 (Luarte et al, 2016). Although, these different neurodegenerative diseases show different entities and pathogenesis, the aggregation of misfolded proteins is not the only thing they have in common. These neurodegenerative diseases also show an overload of protein within the protein degradation pathway also known as autophagy, impaired homeostasis and a

dysfunction is neurons (Hartmann et al, 2017). Despite, being different diseases and having different disease drivers the aggregation of proteins contributes to other impaired physiological functions seen in neurodegenerative diseases. sEVs are known to be secreted by many cell types within the CNS, including; oligodendrocytes, neurons and astrocytes and therefore, sEVs are thought to contribute to the pathogenesis and transmission of disease (Hartmann et al, 2017).

The spread of misfolded protein species in neurodegenerative diseases like AD, FTD and ALS is still up for debate. Direct cell to cell contact is thought to be one of the ways pathogenic proteins spread throughout the brain however, within the past decade research has shown other mechanisms of pathogenic spread through the use of vesicular transport (Kalani et al, 2014). It is now known that sEVs are involved in the spread and progression of neurogenerative diseases through being highly expressed via cellular prion protein (PrP^C) (Luarte et al, 2016). Within neurodegenerative diseases, the PrP^C has at least two known functions; it contains a substrate for generating the pathological prion protein as well as being a key role within the pathophysiological of disease (Hartmann et al, 2017). The $PrP^{C.}$ binds to the neurotoxic Aβ which are associated with the initiation and progression of AD (Hartmann et al, 2017).

sEVs are the hope for an early diagnosis, monitoring disease progression and treatment optimisation in neurodegenerative diseases (Liu et al, 2019). Research has shown that disease related proteins are present within sEVs isolates from plasma and CSF samples of patients with AD, which could be used as an AD biomarker (Pluta & Ulamek-Koziol, 2019). Furthermore, other research shows that sEVs derived from neurons of a patient with AD contained autolysosome proteins and changes in LAMP1, changes in autophagy markers could also be an early detection method (Goetzl et al, 2015). Detected via sEVs derived from patient plasma samples, it was found that there were changes of tau and Aβ levels and therefore, is a good indicator of sEVs being a promising early detection method as well as being able to monitor the disease progression (Goetzl et al, 2015).

Majority of the research carried out to investigated use of sEVs in neurodegenerative diseases use sEVs derived from serum, blood, plasma & CSF which are mostly considered to

be non-invasive and perfect use for a biomarker. However, sEVs derived from brain tissue could be more informative given this is where the majority of disease associated changes are taking place, and there may be some peripheral masking of cargo change when investigating peripheral tissues only. This research projects uses sEVs from what is considered a minimal invasive method (fibroblast skin cells) in the periphery as well as human brain tissue from the site of damage. There are only a few studies worldwide investigating brain tissue derived sEVs, Andrew Hill's group in Australia being the main one (Hill, 2019) and this method had previously not been used in Lace Lab. Being able to isolate sEVs from human brain tissue will seed an abundance of future work where sEV cargoes can be studied in relation to the different neurodegenerative diseases.

1.12 Fibroblast skin cells

Skin-derived fibroblast cells (commercially available from Corriel Cell Repositories) from AD patient and similar aged non-diseased control individuals could be a potential source of sEVs where cargoes can be studied. These cells are obtained by a 2mm punch skin biopsy and these cells are particularly useful because it is a procedure is a less invasive procedure and can be carried out whilst patient is alive unlike a brain autopsy. These are also commercially available. These cells are well characterised and are known to harbour many disease associated changes such as oxidative stress, oxidative DNA damage and impaired oxidative metabolism (Ramamoorthy et al, 2012). To date, sEVs have not been isolated or studied in AD patient derived fibroblasts.

1.13 Aims of study

This study aimed to develop methods to isolate sEVs from both AD patient derived fibroblasts as well as AD patient human brain tissue. This study aimed to characterise the sEVs via Western Blotting and nanoparticle tracking analysis and to see if AD and autophagy associated proteins could be detected in as cargoes within the sEVs.

2.1 Materials and methods

2.2 Human brain tissue and patient derived fibroblasts

There were two different models used in the project, human frontal brain tissue from a range of Braak groups as well as commercially available human derived fibroblast skin cells obtained from AD and aged-controlled patients.

The human brain tissue was donated from Manchester Brain Bank and all work was carried out under the Human Tissue Act 2004. Human brain tissue was sourced from the Manchester Brain Bank which part of the Brains for Dementia Research consortium. Brains were collected from donors and processed and stored, before being provided to the University of Salford to conduct experiments after a successful tissue request submission. For this project there was access to 4 age related control cases at braak group 0-II and 4 AD cases at braak group V-VI from the frontal cortex further details are summarised in table 1 including their age, gender and AD status.

Table 1; Frozen human brain sections were used to isolate sEVs from human brain tissue from both AD groups and controlled groups across different braak groups.

Brain sample	AD diagnosis	Braak stage	Age	Gender
15.26	No	0-1	82	F
15.31	No	1-2	91	M
15.01	No	1-2	90	M
15.30	No	2	90	F
17.07	Yes	5	80	M
15.20	Yes	6	71	M

15.21	Yes	6	79	F
15.08	Yes	6	61	F

There was a total of 40 human brain sections stained with antibody p62, the human brain sections were formalin-fixed, wax embedded sections from the temporal cortex of 17 cases in Braak stage 0-II, 8 cases in Braak stage III-IV and 15 cases in Braak stage V-VI.

There was a total of 32 human brain sections stained with antibody AT8, the human brain sections were formalin-fixed, wax embedded sections from the temporal cortex of 12 cases in Braak stage 0-II, 8 cases in Braak stage III-IV and 12 cases in Braak stage V-VI.

The fibroblast skin cells were bought from Coriell. Cells had been screened for known hazards prior to purchase. There were 2 AD cases and 1 controlled case of fibroblast cell lines which were available during this project, further information of the cell lines include age and gender of the donors are summarised in table 2.

Table 2; All the human fibroblast cell lines were ordered from Coriell and were checked prior for any safety hazards.

Cellular model & catalogue number	Supplier of samples	Description of donor	Affected with AD
Human skin derived fibroblasts - AG06869	Coriell	60-year-old Female.	Yes - moderate dementia.
Human skin derived fibroblasts - AG07872	Coriell	53-year-old Male.	Yes - Moderate dementia.
Human skin derived fibroblasts -AG08125	Coriell	64-year-old Male.	No - control sample.

In the first instance due to the preciousness of the human brain tissue samples there was a limited amount of tissue available, the human brain tissue with the maximum weight was

used to validate the presence of sEVs. This was conducted using 2 AD samples and 2 controlled samples until the methodology was optimised and positive results were obtained other tissue samples were then used for further experiments.

Local ethical approval from the University of Salford for this project was granted and since the tissue had been collected by the Manchester Brain Bank, the ethical approval for use, collection and storage was granted to them (REC 09/H0906/52).

2.2.1 Cell culture

AD and age-matched control fibroblasts (see table 2) were used to investigate if sEV could be isolated and characterised from these commercially available, peripherally derived cells.

2.2.2 Thawing out cells

Cells *(Coriell)* were thawed quickly using a 37°C water bath then 1ml pre-warmed media *(Fisher scientific)* was added to dropwise to the cells before centrifuging at 400xg for 5 mins. Cells were placed in an incubator at 37°C and 5% CO_2.

2.2.3 Sub-culturing

Cells were sub-cultured when reached a confluency of above 70%. Cells were washed twice with phosphate-buffered saline (PBS) *(Fisher scientific)* before trypsinizing the cells with 1mL of EDTA-trypsin *(Fisher scientific)*. Cells were centrifuged at 400xg for 4 minutes before being transferred into fresh pre-warmed media and were stored the incubator at 37°C and 5% CO_2.

2.2.4 Cryopreservation

Cells were centrifuged at 400g for 4 minutes and before the cell pellet was resuspended into freezing media (90% Fetal bovine serum (FBS) *(Fisher scientific)* with 10% Dimethyl Sulfoxide (DMSO) *(Fisher scientific)* as a cryoprotectant and aliquoted into labelled cryovial and transferred into Nalgene™ freezing container and stored in the -80°C freezer for 24 hours before, cryovials were transferred and stored in liquid nitrogen (below -190°).

2.2.5 Establishing fibroblast cell culture;

Fibroblast skin cells are known to lose their original phenotype during sub-culturing, fibroblast cells are not recommended to be used experimentally beyond passage number 14. As the passage number increases there becomes an increase in gene expression and therefore, experiments were carried out within one passage of one another to ensure consistency and accuracy (Hernot et al, 2020).

2.2.6 Maintenance and growth of skin derived fibroblast skin cells

The commercially derived fibroblasts (Control & AD cell lines) were cultured in Minimum Essential Medium (MEM) 500mL, supplemented with 15% Foetal Bovine Serum (FBS) 75mL, 1% MEM non-essential amino acids 5mL *(Fisher scientific)*, 1% 2mM L-Glutamine 5mL *(Fisher scientific)*, 1% of penicillin/streptomycin 5mL *(Labtech).* The fibroblast cells were kept at 37 °C with 5% CO_2 in the incubator to grow.

2.3 Preparation for sEV isolation

The media was changed when the cell confluency reached around 60% and the media was added and left for 3 days prior to the sEV isolation. After the 3 days the cell culture supernatant was used for the sEV isolation. A cell count was performed using a haemocytometer after the 3-day duration period; 1:1 dilution (20µl) of cell pellet and trypan blue solution *(Sigma)* was used to calculate the cell viability ensuring there were at least 8 million cells within the flask.

2.3.1 sEVs - Purification of whole cell homogenate

sEVs from cell culture supernatants were isolated by ultracentrifugation. Cells were grown in MEM medium with exosome depleted FBS *(Thermo Fisher Scientific).* The media was collected and centrifuged at 300 × *g* for 10 minutes before being centrifuged at 10,0000 × *g* for 48 minutes at 4 °C. The exo-spin buffer *(Cell guidance)* was added at 2:1 ratio (Supernatant: exo-spin buffer) and was left overnight at 4 °C. Then, ultracentrifuged at 10,000 × *g* for 96 minutes. The EV pellet was washed with 250 µL of PBS followed by Size

Exclusion Chromatography (SEC) to isolate the sEVs from the sample - the SEC column was spun down at 50 x g for 60 seconds to receive the purified sEV sample.

2.3.2 sEVs -Purification of brain homogenate

Frozen human brain tissue (stored at -80°C) was sliced on dry ice into 1-2cm long and 2-3mm wide sections using a razor blade. The frozen sections were transfer into 75 U/ml of collagenase type 3 *(Cell signalling)* in Hibernate-E *(Thermo Fisher Scientific)* at 37°C for a total of 20 min, after 5 minutes the tube was gently inverted to mix, after 10 minutes the frozen brain tissue + collagenase type 3 in Hibernate-E were gently mixed using a stripette. The tissue was returned to ice immediately after incubation and PhosSTOP inhibitor *(Thermo Fisher Scientific)* was added to stop enzymatice digestion. The tissue was spun at 300 × *g* for 5 minutes at 4°C. The supernatant was transferred into a fresh tube, spun at 2000 × *g* for 15 minutes at 4°C. The brain homogenate with collagenase (BHC) pellet was resuspended into PBS and stored at -80°C. The supernatant was filtered using a 0.22 μm filter then centrifuged 10 000 x g for 30 min at 4 °C. The 10K Pellet was resuspended into 100 μl PBS and concentrated prior to exo-spin column isolation. The '10K pellet' was placed on top of the column and was centrifuged at 50 x g for 10 seconds, the flow through was discarded. 180μl of PBS was added to the top of the column and the SEC column was spun down at 50 x g for 60 seconds to receive the purified sEV sample (Hill, 2019).

2.4 Protein quantification (BCA assay)

Protein quantification of prepared samples were determined via BCA *(Pierce BCA Protein Assay Kit, Thermofisher, UK)* protein assay and was carried out using the manufacturer's instructions to ensure similar protein loading across experiments. After the working regent was added to the samples in the 96 well plate, the plate was placed on the shaker for roughly 30 seconds before being incubated for 30 minutes at 37°C, with 100 μL of sample + 100 μL of the working reagent. Absorbance was then measured at 562 nm using the Varioskan Lux Multimode microplate reader *(Thermo Scientific).*

2.5 Western Blot

Western Blotting was used to confirm the presence of sEV specific antibodies on sEV isolates derived from both human brain tissue and human fibroblast skin cell associated extractions. The specific antibodies used in the western blots are in table 8.

Samples were prepared using Laemmli loading buffer *(Biorad)* and heated at 70°C for 10 minutes. The samples were separated by polyacrylamide gel *(Biorad)* electrophoresis. The polyacrylamide gel was transferred to polyvinylidene fluoride membranes *(Millipore, Billerica, MA, United States)* for 1 hour and a half at 4°C. The membrane was blocked in 5% bovine serum albumin (BSA) *(Thermo Fisher Scientific)* at room temperature for 1 hour. The blots were probed at 4°C overnight with diluted primary antibodies. The membrane was rinsed three times with Phosphate Buffered saline + Tween 2.0 *(Thermo Fisher Scientific)* (wash buffer) (each time for 10 min), before being incubated with horseradish peroxidase-linked anti-mouse immunoglobulin G *(Cell Signalling, 1:5000, 7076S)* at room temperature for 2 hours. After, the secondary antibody incubation the membrane is washed again using wash buffer (3 x 10 minutes) before imaging with Super signal West Atto ultimate sensitivity chemiluminescent Substrate *(Thermofisher Scientific)* 250 µL of Reagent A and Reagent B mixed and left on the membrane for 2 minutes before imaging on the GBOX.

Table 3; Ingredients to make 10% Polyacrylamide gel.

Resolving gel - 10ml		Stacking gel- 4ml	
ddH_20	4mL	ddH_20	2.7mL
Acrylamide (30%)	3.3mL	Acrylamide (30%)	670µL
Tris-HCL [1,5M] pH8.8	2.5mL	Tris-HCL [1,5M] pH6.8	500µL
SDS [10%]	100µL	SDS [10%]	40µL
APS [10%]	100µL	APS [10%]	40µL
TEMED	4µL	TEMED	4µL

Propanol was added on top of the resolving gel to get rid of any bubbles between the glass plates and to create anaerobic conditions for polymerisation.

2.5.1 Buffer ingredients & antibodies used for western blot

Sample loading buffer = Laemmli loading buffer [4X] *(Biorad).*

Wash buffer = 0.1% Tween 20 diluted in PBS.

Table 4; Ingredients to make running buffer for SDS-page [x10]; End volume 1L - To make [x1] dilute by 10 with ddH_2O.

Material	Concentration [mM]	Weight (g)
Tris Base	250 mM	30.3
Glycine	192mM	144.0
SDS	1%	10.0

Table 5: Ingredients used to make transfer buffer for semidry transblotting [x1].

Material	Concentration [mM]	Weight (g) / Volume (mL)
Tris Base	250 mM	3.03
Glycine	192mM	14.4
Methanol	20%	200mL

Table 6; Mild stripping buffer ingredients - to strip and re-probe the membrane for another primary antibody of interest.

Ingredients	Weight (g)/ Volume (mL)
Glycine	1.5g
SDS	0.1g
Tween 20	1mL
ddH_20	85mL

Hydrochloric Acid [1 Molar]	15mL

Table 7; All the primary antibodies used in the western blots, to confirm the presence of extracellular vesicles and to confirm the presence of pathogenic protein markers linking to AD.

Target;	Supplier;	Host species;	Classification	Dilution.
CD9	Fisher Scientific (15328354)	Mouse	Monoclonal	1:1000
CD63	Fisher Scientific (15410554)	Mouse	Monoclonal	1:2000
Flotillin-1	Proteintech (67968-1-lg)	Mouse	Monoclonal	1:1000
Calnexin	Proteintech (66903-1-lg)	Mouse	Monoclonal	1:1000
Phospho-Tau (Ser202, Thr205) (AT8)	Fisher Scientific (MN10120)	Mouse	Monoclonal	1:500
P62 (SQSTM1)	Biosciences (610832)	Mouse	Monoclonal	1:500
Beta Actin	Fisher Scientific (15739)	Mouse	Monoclonal	1:1000

Antigens were detected through the use of HRP-conjugated secondary antibody anti-mouse IgG followed by super signal West Atto ultimate sensitivity chemiluminescent substrate. Visualisation was performed using a Syngene, G:BOX Chemi XL XTI6 imager with a transilluminator lab gel imaging system.

Once the presence of sEVs had been established, the presence of AT8 (tau) *(Thermo Fisher Scientific)* and P62 *(Biosciences)* were explored as a pilot study to investigate if these could be detected within the sEV derived isolates. In this preliminary investigation 3 control cases and 3 AD cases were used. Both of the antibodies were used at 1:500 dilution respectively.

2.6 Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis was performed using ZetaView *(Particle Matrix, Germany).* The NTA technique measures the size and number of particles based on the random movement called "Brownian motion" within the sample. All particles within the sample are measured - not just the biological particles. Samples were diluted in PBS in the ratio 1:1000. The sample was pushed into the cell and the raw data was collected and analysed for size distribution.

2.6.1 Fluorescent Nanoparticle tracking- Cell Masked Orange

In order to measure the biological particles of the sample, the sample dilution was measured to ensure there was at least 200 particles per frame from the scatter mode. Next, the lipophilic membrane dye Cell mask orange (*CMO, Invitrogen)* was prediluted in PBS at a ratio of 1;10,000. Using the prediluted CMO dye the dye and sample were mixed at 1:10 ratio and left for half an hour at room temperature out of direct sunlight. After an hour, the sample and dye were diluted into 1mL of PBS 1:1000 dilution and inserted into the cell. The minimum the cell will hold to receive an accurate reading is 1mL. The instruments works on the principle of scanning technology and the use of a single laser at a wavelength of 520 nm with a 550 nm long-wave pass filter once the sample has been read the raw data of size and concentration was received and evaluated, these results are showing the biological particles present within the sEV sample before testing for the tetraspanins antibodies, CD9, CD63 & CD81 *(Biolegend).*

2.6.2 Fluorescent Nanoparticle tracking- antibody markers; CD9, CD63 & CD81

In order to measure the biological particles of the sample that contain the tetraspanins antibodies; CD9, CD63, CD81 (which would confirm the presence of sEVs), the sample dilution was measured to ensure there was at least 200 particles per frame from the standard NTA from the scatter mode. The antibodies CD9 *(PE/Dazzle™ 594 Antibody, Biolegend),* CD63 *(PE/Dazzle™ 594 Antibody, Biolegend)* and CD81 *(PE/Dazzle™ 594 Antibody, Biolegend)* were prediluted into PBS 1:100. The prediluted antibody dye was then mixed with sEV sample 1:10 and left at room temperature out of direct sunlight for a minimum of two hours. After the incubation period, the sample and dye were diluted into

1mL of PBS and inserted into the cell to be analysed. The instrument works on the principle of scanning technology and the use of a single laser at a wavelength of 520 nm with a 550 nm long-wave pass filter once the sample has been read the raw data of size and concentration were evaluated.

Table 8; Antibodies used on Nanoparticle tracking analysis to confirm the presence of small extracellular vesicles isolated from fibroblast skin cells using Exo-Spin (Cell guidance) isolation technique.

Antibody	Supplier	Isotype	Dilution
CD9	Biolegend (312118)	Mouse	1:100,000
CD63	Biolegend (353022)	Mouse	1:100,000
CD81	Biolegend (349520)	Mouse	1:100,000

2.6.3 fNTA optimisation steps

The first fNTA antibody experiments were attempted under the following conditions suggested by the manufacturer stating the incubation period of a minimum of 1 hour. However, due to the lack of particles detected experiments were repeated, adjusting the incubation period to a minimum time duration of 2hrs. After the second lot of experiments, the antibody dilution was adjusted and trialled to see if this could increase the number of particles detected with the CD markers. Although, the final antibody dilution needed to be a 1:100,000 dilution, the dilutions in-between (dye: PBS and the sample: PBS) were altered to potentially increase the particles detected.

2.6.4 NTA statistical analysis

To investigate the number of particles and the size of the particles within the AD and control groups, an unpaired t-test was performed. with results being interpreted as being significant if the p value was less than 0.05.

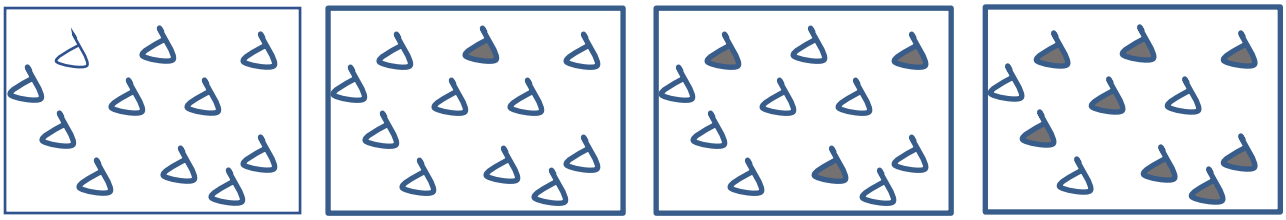
2.6.5 P62 Immunohistochemical staining;

A preliminary study was conducted to explore p62 detection in human brain extracts via western blotting. To compliment this, variation in p62 staining across Braak groups were also conducted, using formalin-fixed, wax embedded sections from the temporal cortex of 17 cases in Braak stage 0-II, 8 cases in Braak stage III-IV and 15 cases in Braak stage V-VI.

Sections had been previously stained as follows. Sections were dewaxed in Histoclear (National Diagnostics) via two five-minute incubations, then were rehydrated via sequential five-minute incubations in alcohols of different % concentrations (100%, 100%, 95% and 75% to distilled water). Endogenous peroxidase was blocked using 3% H_2O_2 in methanol for 20 mins and then Heat Induced Epitope Retrieval (HIER) was performed via microwaving sections in pH 6.5 0.01M tri-sodium citrate buffer for 10 minutes. Endogenous peroxidase activity was blocked via a 30-minute incubation in 3% H_2O_2 in methanol and non-specific binding sites were blocked using 1.5% normal serum from the Vectorstain ELITE kit (Vector Laboratories) for one hour before being incubated with the anti-P62 primary antibody (1:200, mouse, Abcam). Sections were washed in TBST and then slides were incubated for 30 minutes incubation in animal specific anti-IgG secondary antibody (1:200, Vectorstain ELITE). Sections were then washed with TBST and incubated for another 30 minutes in avidin-biotin complex (ABC, 1:25), made up 30 minutes prior as per manufacturer's instructions before a final wash in TBST. Immunopositivity was visualised using 3, 3' Diaminobenzidine (DAB) using DAB tablets (Sigma-Aldrich) made up to 1:100 in PBS 9.6% H_2O_2 .Visualisation of the antigen was conducted with reference to the positive control for a maximum of ten minutes and the same positive control was used to ensure consistency between experimental runs. Development of peroxidase activity was stopped by immersion in tap water. Sections were counterstained in haematoxylin, washed in tap water and dehydrated via 5minute incubations through ascending alcohols concentrations (75%, 95% and 100%, 100%) and cleared using Histoclear, before mounting and cover slipping and Dibutyl Phthalate with Xylene (DPX).

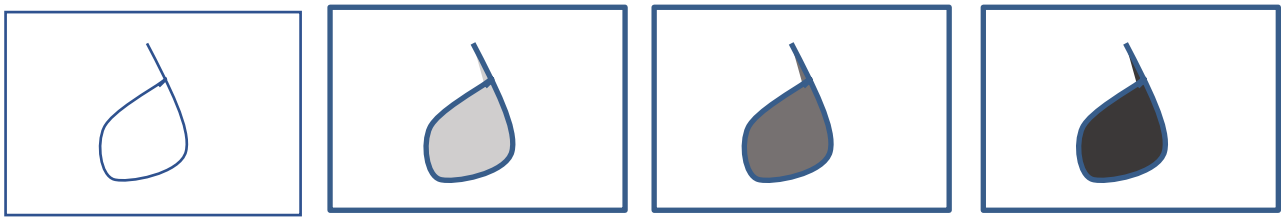
The p62 slides that were available were scored using scoring guide below to investigate the levels of p62 and the intensity of stain within human brain tissue and whether this correlates to the stage of dementia.

1) Score number of cells.



0=no cells 1=<20% mild 2=21-69% moderate 3=70-100% severe

2) Score staining intensity.



No stain Mild stain Moderate stain Intense stain

2.6.6 p62 statistical analysis;

To investigate the significant of the p62 stain intensity across the different braak groups a chi- squared test was preformed using the dichotomised data of the p62 slides available. If the P value is greater than 0.05 the test will be deemed as insignificant however, if the P value is less than 0.05, the test will be deemed as being significant.

3.1 Results

To explore if sEVs could be successfully extracted from commercially available human skin derived fibroblasts from AD patients, a protocol previously used in other cell models was used (Lobb et al, 2015). If sEVs could be derived from human skin cells this would unlock further studies to identify biomarkers for diagnosing AD or FTD and/or in the advancement of understanding of the underlying processes driving disease.

3.2 Fibroblast cell culture

All cell images were taken using primovert microscope at x400 magnification and cells can be seen to have a thin elongated flattened body with some containing webbed end cell bodies, typical of fibroblasts (Ravikanth et al,2011). Some of the cells present within both samples contain thicker cell bodies roughly in the middle of the cell (shown using black arrow) and some cells within the AD sample shown in image B see to be shorter in size with thicker cell body (shown using red arrow) as shown in figure 13.

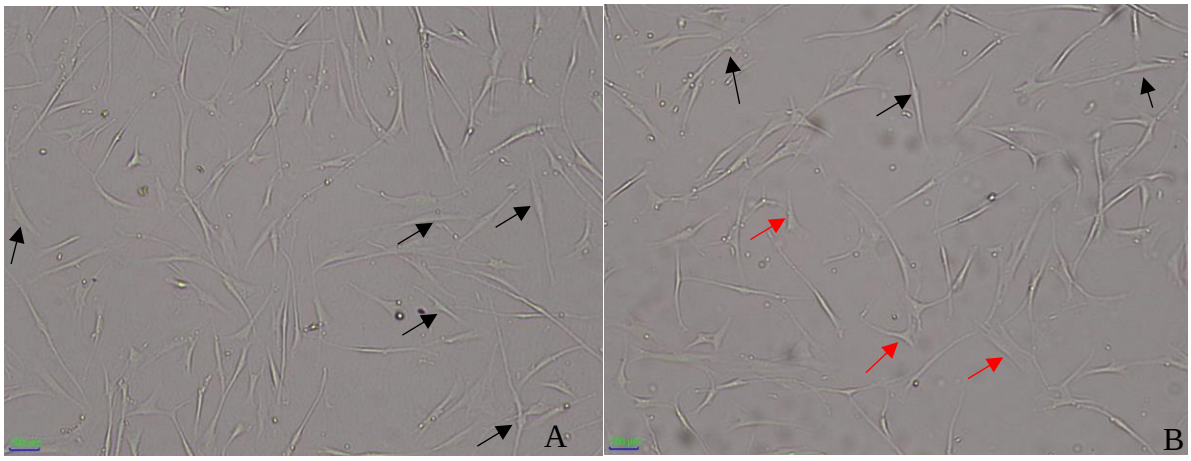


Figure 13; The cell morphology of skin derived fibroblasts from an unaffected donor and an affected donor of AD. A is an image representing the cell line AG08125 a control sample with a confluency of ~70%. B is an image representing the cell line AG05809 an AD sample with a confluency of ~60%.

Despite having some similarities in morphology with the majority of cells having elongated thin cell with webbed cell bodies in both of the samples there are some differences as well (seen in figure 13). Cells within image B seem to be shorter and thicker compared to those cells in image A. In both images the cells seem to be growing over one another despite being adherent cells. It can be however, seen that there are no clear unexpected differences in cell morphology between the AD and control cells.

3.3 Western Blot optimisation

Western blots were used to confirm the presence of sEVs isolated from AD and control fibroblast cell conditioned media, using specific markers CD9 and CD63 which are sEV specific markers. As a starting point, one AD and one control was utilised for optimisation, then an additional AD cell line AG07872 we as introduced in case of disease associated variability that might impact band detection. A positive control is used to show what the

expected result should look like as well as to see if the experiment worked correctly. A positive control is chosen carefully to ensure it will express the antibody of interest. The positive control in this experiment was a cell lysate taken from the whole cell homogenate of the fibroblast skin cells and would have included some intracellular sEVs. In some of the first experiments PBS was used as a negative control given there should be no sEV markers in PBS. A series of optimisation experiments were required to allow detection of the sEV markers in the first instance.

The first part of the optimisation process included exploration of the optimum proteins concentrations when using the manufacturer recommended protein load of 20µg.

Table 9; The protein concentration of samples used in the first Western Blot experiments (µg/µl) as determined by BCA.

Sample	Concentration (µg/µl)
AD1 - Whole cell homogenate	0.2
AD1 - sEVs	0.01
C1 - Whole cell homogenate	0.01
C1 - sEVs	0.01

Table 10; The total protein concentration of the samples isolated and used in the Western Blot's experiments after making fresh samples and changing storage conditions (µg/µl).

Sample	Concentration (µg/µl)
AD1 - Whole cell homogenate	3.04
AD1 - sEVs	0.63
AD1 - Void	NA
C1 - Whole cell homogenate	4.26
C1 - sEVs	0.69
C1- Void	NA

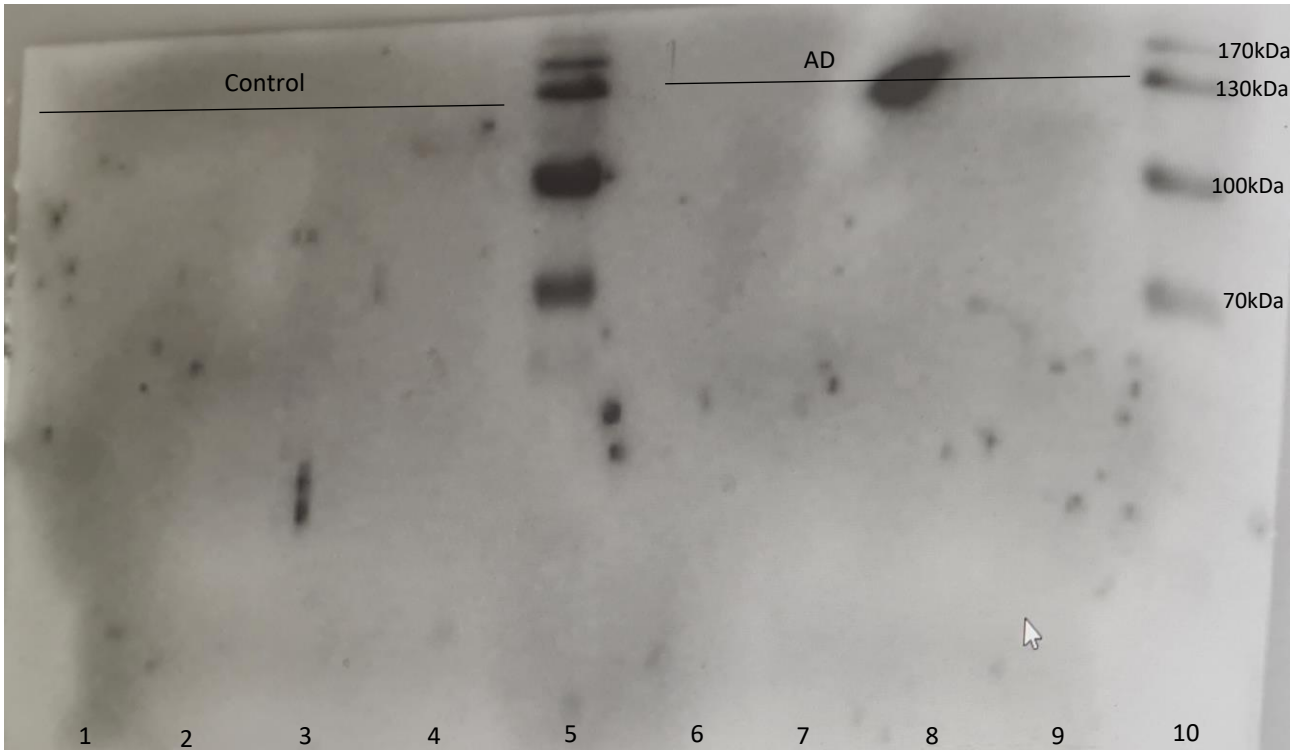


Figure 14; A comparison of loading protein concentrations to allow optimum detection of CD63 (1:2000). Lane 1 - 4 were samples derived from the controlled case and lanes 6-9 were samples derived from the AD case. Lane 1 contained protein fractions from the SEC experiment derived from C1 and Lane 6 contained protein fractions from the SEC experiment derived from AD1. Lane 2 (C1) and lane 7 (AD1) were filled with PBS also known as the 'flow-through' from the SEC experiment. Lane 3 cell derived sEVs from the controlled case and lane 8 cell derived sEVs from AD case. Lanes 4 (C1) and lane 9 (AD1) were whole cell homogenates used as positive controls. Lanes 5 and 10; molecular weight marker.

A band was detected in lane 8 and were seen of 130kDa, but for positive detection of CD63 a smeary band of 35-65 kDa would be expected. No bands were detected in either of the positive controls (lanes 4 and 9) which means the protein concentrations used were not sufficient to allow CD63 detection. As expected, no bands were identified in the negative controls (lanes 2 & 7). Therefore, for this experiment it does not seem to be the most promising protein loading amount to allow optimum detection. The results of the experiment also showed that there could possibly be a problem with the sample preparation or general western blot process due to the positive controls not being detected either and therefore, further optimisation was needed. The negative control was negative however, based on the result of this experiment it shows that sEV's were not successfully extracted.

Due to the lack of positive controls presence in the first experiment, the second part of the optimisation process was to change the antibody to investigate whether there was a problem with the antibody, or if it was related to sample preparation.

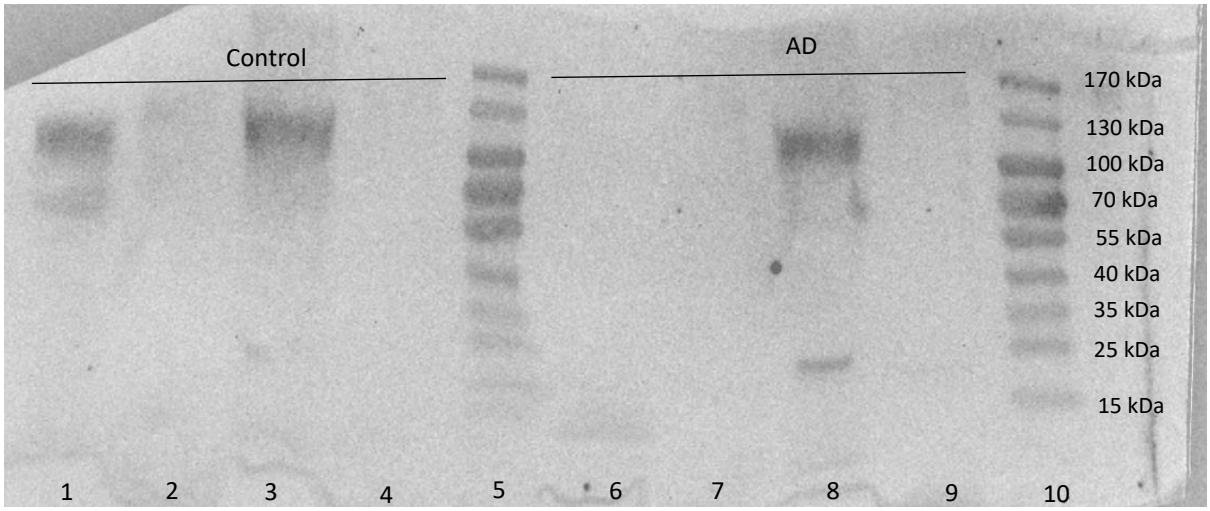


Figure 15; A comparison of loading protein concentrations to allow optimum detection of CD9 (1:1000) from fresh sEV samples. Lane 1 - 4 were samples derived from the control case (AG08215) and lanes 6-9 were samples derived from the AD case (AG06869). Lane 1 contained protein fractions from the SEC experiment derived from C1 and Lane 6 contained protein fractions from the SEC experiment derived from AD1. Lane 2 (C1) and lane 7 (AD1) were filled with PBS also known as the 'flow-through' from the SEC experiment. Lane 3 cell derived sEVs from the controlled case and lane 8 cell derived sEVs from AD case. Lanes 4 (controlled case) and lane 9 (AD case) were whole cell homogenates used as positive controls. Lanes 5 and 10 were the molecular weight marker.

Bands were present in lane 1, 3, and 8 at the 130kDa region which is not the region which represents CD9. However, also in lane 8 there is a faint band at the 25kDa band which is the region that represents CD9. Due to the positive controls not showing in lane 4 and 9, it cannot be justified that the band in lane 8 is CD9. There were no bands present in lane 2 and 7 as these were negative controls.

CD9 was not expressed in the positive controls therefore, this experiment did not work. The samples were stored in PBS -80 degrees and therefore, were not lysed open. The next optimisation step included fresh samples being prepared and samples being stored in RIPA after a 30 minute agitation. The protein concentration did not change.

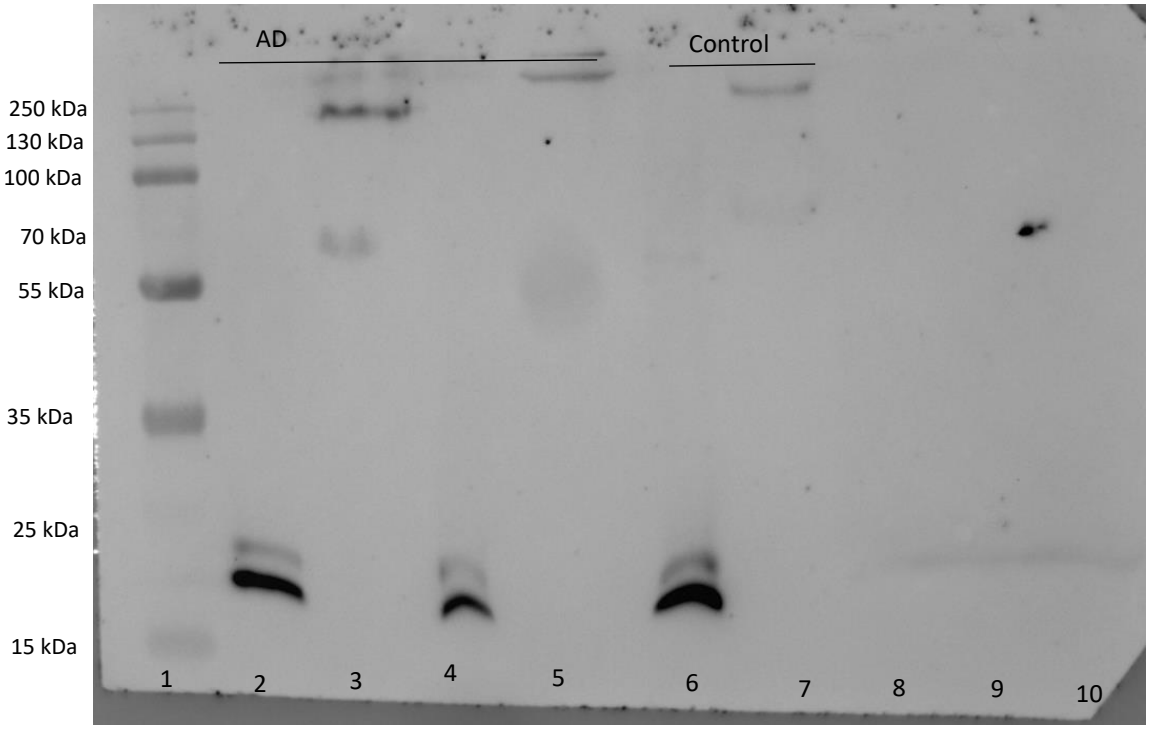


Figure 16; To investigate the storage conditions of freshly made cell lysates in RIPA compared to PBS to allow optimum detection of CD9 (1:1000). Lanes 2 and 3 were derived from cell line AG06869 an AD case, lanes 4 and 5 were derived from cell line AG07872 another AD case and lanes 6 and 7 were obtained from a control case AG08125. Lane 1 contained molecular weight marker. Lane 2 contained whole cell homogenate from AD case AG06869 and Lane 3 contained cell derived sEVs from AD case AG06869. Lane 4 contained whole cell homogenate from AD case AG07872 and lane 5 contained cell derived sEVs from AD case AG07872. Lane 6 contained whole cell homogenate from control case AG08125 and Lane 7 contained cell derived sEVs from control case AG08125. Lanes 8-10 contained PBS as negative controls.

There were bands present in lanes 2, 4 and 6 which were the positive controls at the region of 25kDa which represents the correct region of the CD9 antibody. There were no bands present in lanes 8-10 as this was a negative control. There were no bands present in lanes, 3, 5 and 7 and therefore, is an indication that CD9 was not present and that sEVs were not successfully extracted.

Further optimisation steps occurred to investigate the optimum antibody dilution as well as adding DDT to the LDS sample loading buffer to try and resolve the problem of the sEV samples being stuck at the top of the membrane this optimisation step includes 3 membranes.

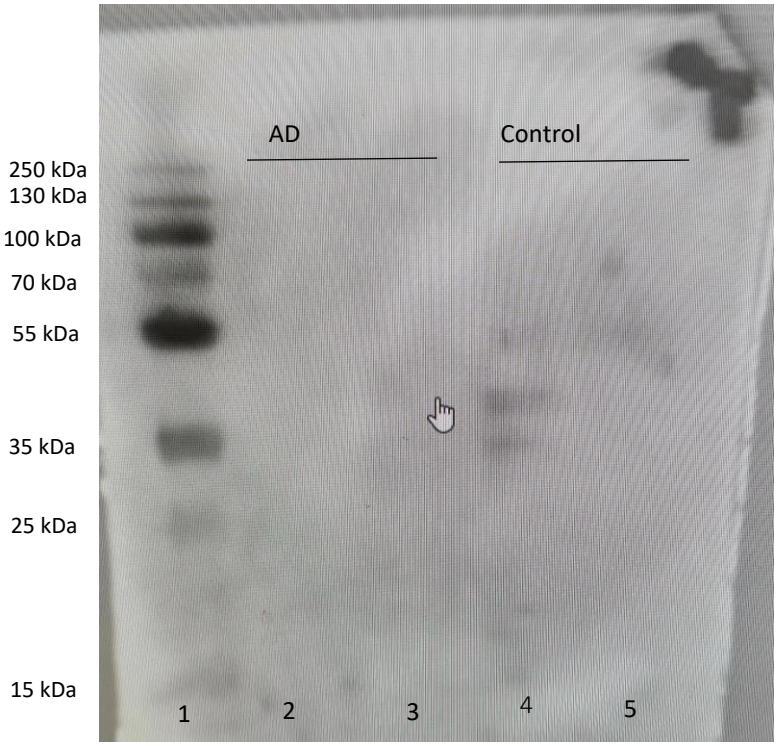


Figure 17; To compare different CD9 dilutions (1:500) and the effect of DDT within the LDS sample buffer. Lane 1 contained the molecular weight marker. Lane 2 cell homogenate derived from AD case AG06869. Lane 3 cell derived sEVs from AD case AG06869. Lane 4 cell homogenate derived from control case AG08125. Lane 5 cell derived sEVs from AD case AG08125.

In this experiment no bands were present in either of the lanes not even in the positive control lanes and therefore, is not indication of CD9. The sEV lanes were also absence of bands and therefore indicates that sEVs were not extracted. These samples contain DDT which could be the reason for a messy blot containing a lot of background noise.

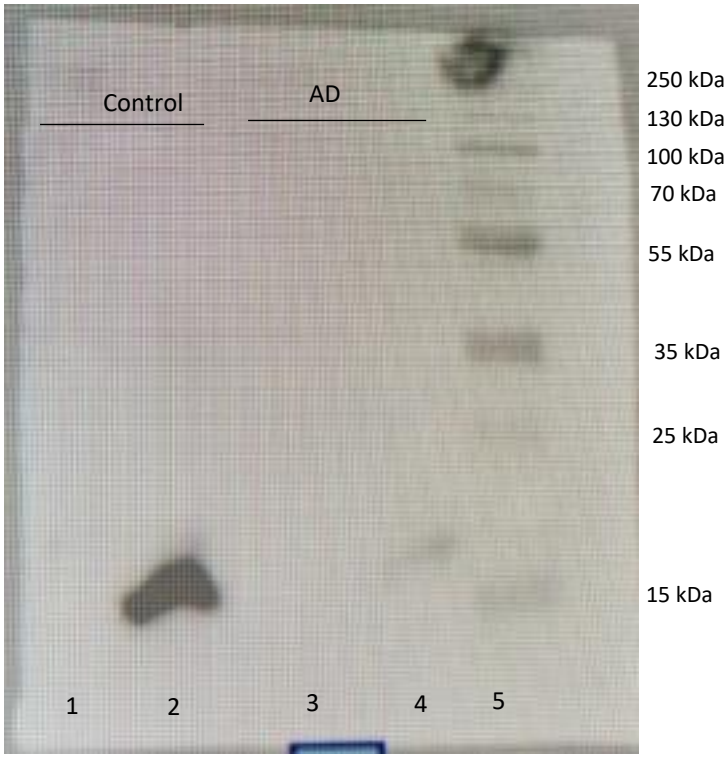


Figure 18; To compare different CD9 dilutions (1:1000) as well as to investigate the use of LDS sample buffer without DDT. Lane 1 cell derived sEVs from AD case AG08125. Lane 2 cell lysate derived from control case AG08125. Lane 3 cell derived sEVs from AD case AG06869. Lane 4 cell lysate derived from AD case AG06869. Lane 5 contained the molecular weight marker.

There were no bands present in lanes 1, 3 and 4. There is a band present in lane 2 which is a positive control however, it is not a clear whether it is an indication of CD9 as the molecular weight is closer to 15kDa than it is to 25kDa which would be a better indication of CD9. Lane 4 is another positive control however, there is no band detected. Lane 1 and 3 contained the sEV derived sample however, due to the lack of bands present it is clear that there is no sEVs present and therefore, shows that sEVs were not successfully extracted from the fibroblast skin cells.

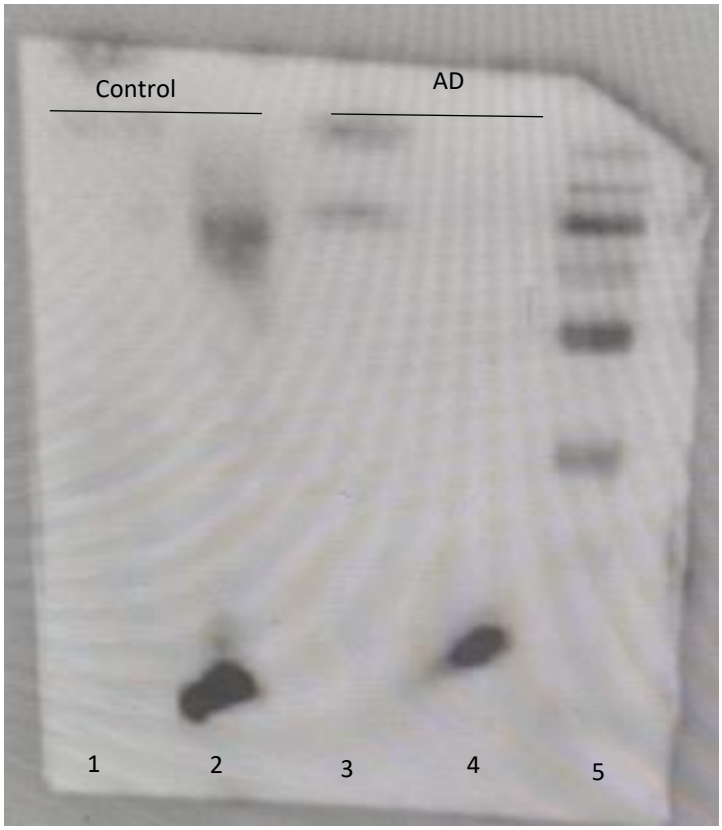


Figure 19; To compare different CD9 dilutions (1:500) and without DDT within the LDS sample buffer. Lane 1 cell derived sEVs from AD case AG08125. Lane 2 cell lysate derived from control case AG08125. Lane 3 cell derived sEVs from AD case AG06869. Lane 4 cell lysate derived from AD case AG06869. Lane 5 contained the molecular weight marker.

In lanes 1 and 3, no bands were detected but in lanes 2 and 4 bands, the positive controls were identified below the 35kDa region, which is an indication of CD9 detection. CD9 has a molecular weight of roughly 24kDa. The positive controls shown up at the correct region however, the sEV samples did not present a band and therefore, are unable to say that sEVs derived from human fibroblast skin cells were successful.

There were no negative controls used in this experiment, the membrane was split in half to test different antibody dilutions and sample loading buffer conditions as at this point, levels of sEV extract were running low. The results from these optimisation steps show that CD9 1:1000 was successful dilution factor despite not obtaining a result in the skin-derived sEV fraction, it also shows that the present of DDT did not help the sEVs migrate throughout the gel and only increase the background noise which made the blot messy.

Despite carrying out various optimisation steps, there was no positive sEV results from the cell lines which demonstrates challenges in studying sEV's from skin-derived fibroblasts.

Given the challenges trying to demonstrate the presence of sEV's derived from AD and control patient derived fibroblasts, and the time/cell constraints associated with the project, the next part of the work was to attempt to extract sEV's from human brain tissue.

3.4 Human brain tissue sEV Characterisation

This section was carried out alongside 2 other PhD students Toby Aarons and Joseph Morgan using the sEV isolation method adapted by Prof Andrew Hill (Hill, 2019) as described in chapter 2.1. The experiments were completed equally by the group by completing different steps of the human brain isolation and characterisation steps to ensure preservation of the human tissue. However, the results from figures 26-31 and figures 37-39 were carried out independently.

The purpose of the following experiments was not to quantify the amount of each marker but was to qualitatively note the presence/absence of each marker to identify if sEV's had indeed been successfully isolated. Two different methods were used to investigate the presence and absence of sEVs within samples and were confirmed with the use of positive and negative controls. The positive controls in the following experiments consist of brain homogenate with collagenase (BHC) or whole cell homogenate (cell lysates from AG07872 - AD cell line and AG08125 control cell line). The positive controls are samples which are known to have the presence of the antibodies detected, as well as ensuring the experiments have worked correctly. The protein load of each of the sEVs samples were 20µg in the first instance, and the protein concentration of the BHC's and cell lysates were 10µg.

The initial experiments were carried out using brain tissue samples from 2 x AD and 2 x control with case selection for these optimisation studies being based on where that was a larger mass of tissue available and to preserve tissue from cases where there was only a small mass available.

.3.4.1 Human brain tissue characterisation - Western Blots

The antibodies used within the Western blots to confirm the positive presence of sEVs were CD9, CD63, Flotillin-1 whereas Calnexin, an endoplasmic reticulum protein marker, was used as a negative control due to being detected in whole cells and expected to be absent in sEVs in samples. 3 AD and 3 control cases were used in these characterisation studies. Whole cell homogenate from 1 x control cell line case (AG08125) and 1 x AD cell line case (AG07872) were also used in these experiments for comparison.

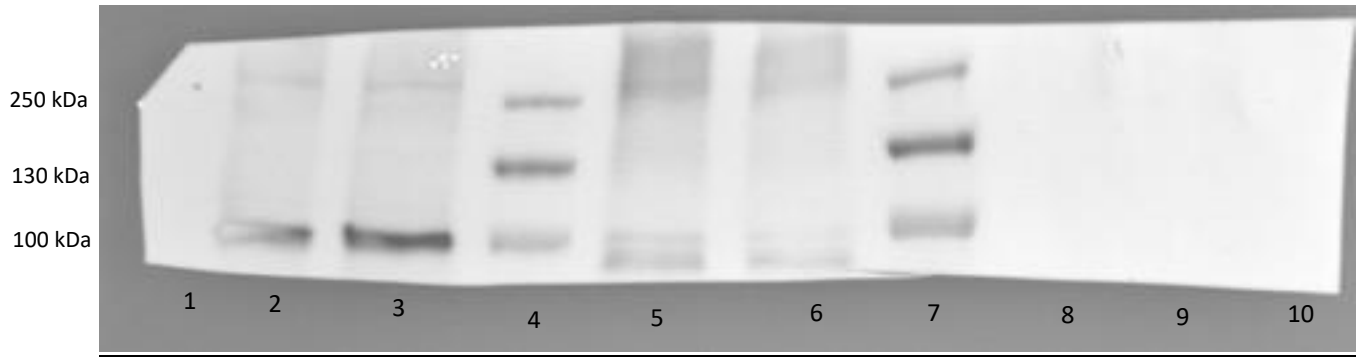


Figure 20; To investigate the presence of calnexin (1:1000) within the following samples; sEV derived from human brain tissue, cell homogenates and brain homogenates. Lanes 1 and 10 contained loading buffer as a negative control. Lane 2 fibroblast homogenate AD AG07872. Lane 3 fibroblast homogenate control AG08125. Lane 4 and 7 molecular weight markers. Lane 5; Human brain homogenate AD (15/08) Lane 6 Human brain homogenate control (15/30) Lane 8 Human brain sEV extract AD (15/08). Lane 9, Human brain sEV extract control (15/30).

In lanes 2, 3, 5 and 6 there were bands identified below the 100kDa region which is an indication of calnexin. There were no bands present in the human brain derived sEVs in lanes 8 and 9, as calnexin is a negative control for sEVs and therefore, should be absent in lanes 8 & 9. The positive controls in lanes 2 and 3 (whole fibroblast cell homogenate) and lanes 5 & 6 (BHC) were positive. Lane 1 and 10 were negative. This evidence shows that sEVs were fraction was not contaminated by other membrane bound structures such as the endoplasmic reticulum.

This membrane was split into 2, to probe for 2 different antibodies. The top of the membrane was cut just above 70kDa to probe for calnexin, the bottom half was cut and probed for CD9 seen in

figure 23. Bands in lane 5 and 6, BHCs derived from human brain tissue show a slightly lower band compared to the whole cell homogenate in lanes 2 & 3.

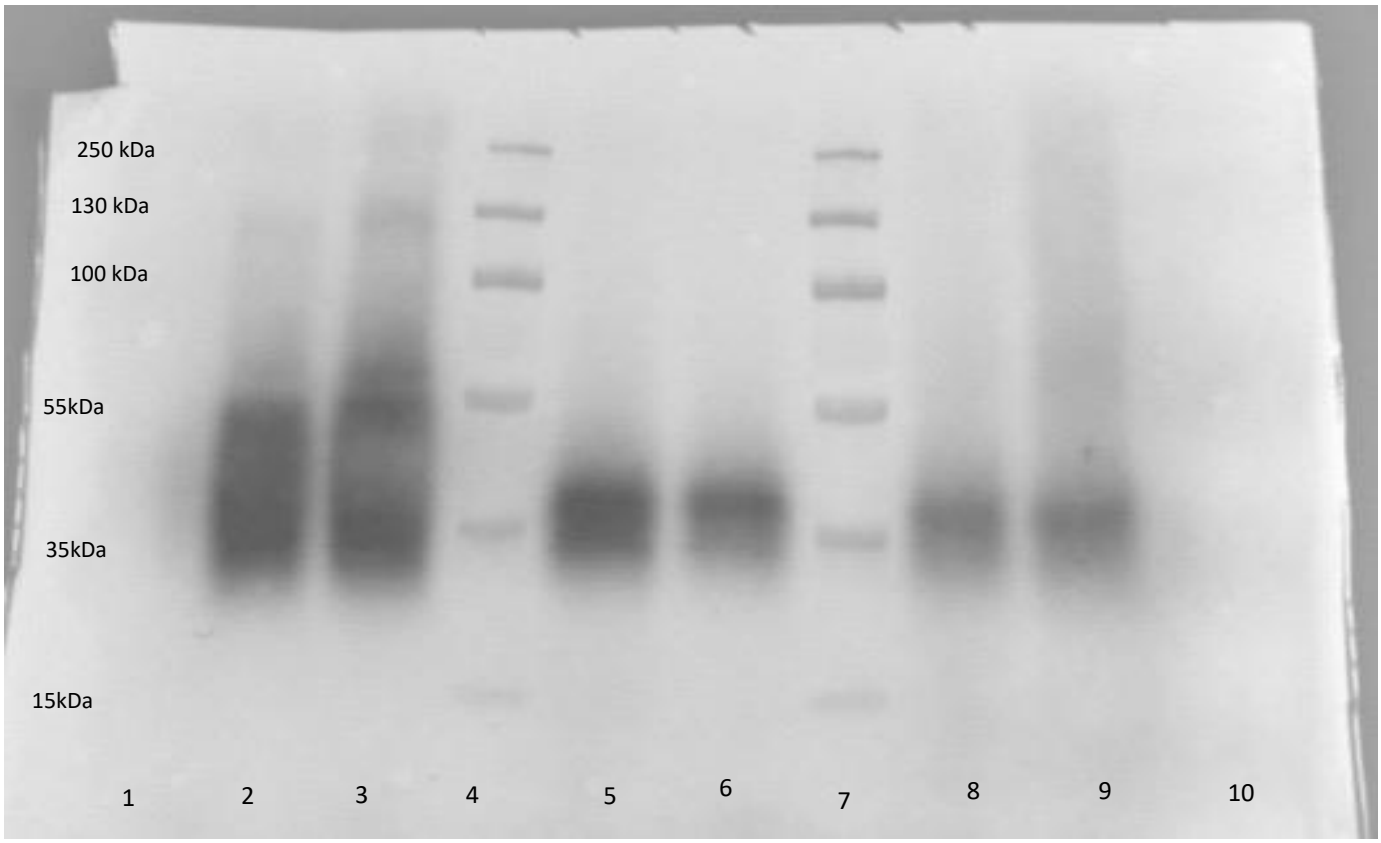


Figure 21; To investigate the presence of CD63 (1:2000) within the following samples; within the following samples; sEV derived from human brain tissue, cell homogenates and brain homogenates. Lanes 1 and 10 contained loading buffer as a negative control. Lane 2 fibroblast homogenate AD AG07872. Lane 3 fibroblast homogenate control AG08125. Lane 4 and 7 molecular weight markers. Lane 5; Human brain homogenate AD (15/08) Lane 6 Human brain homogenate control (15/30) Lane 8 Human brain sEV extract AD (15/08). Lane 9, Human brain sEV extract control (15/30).

In lanes 2, 3, 5, 6, 8 & 9 there were smeary bands identified at 35kDa region which is expected, CD63 shows a band between 35kDa and 55kDa with the nature of the band being a smear. This is a clear indication of CD63. The positive controls in lanes 2 and 3 (whole cell homogenate) were a much darker and bigger smear compared to the other positive controls (BHC) in lane 5 and 6. Lane 1 and 10 were negative controls and therefore, no bands were present as expected. sEVs derived from human brain tissue in lanes 8 and 9 contained a smeary band at 35kDa which indicates CD63 and therefore indicates the successful extraction of sEVs from human brain tissue.

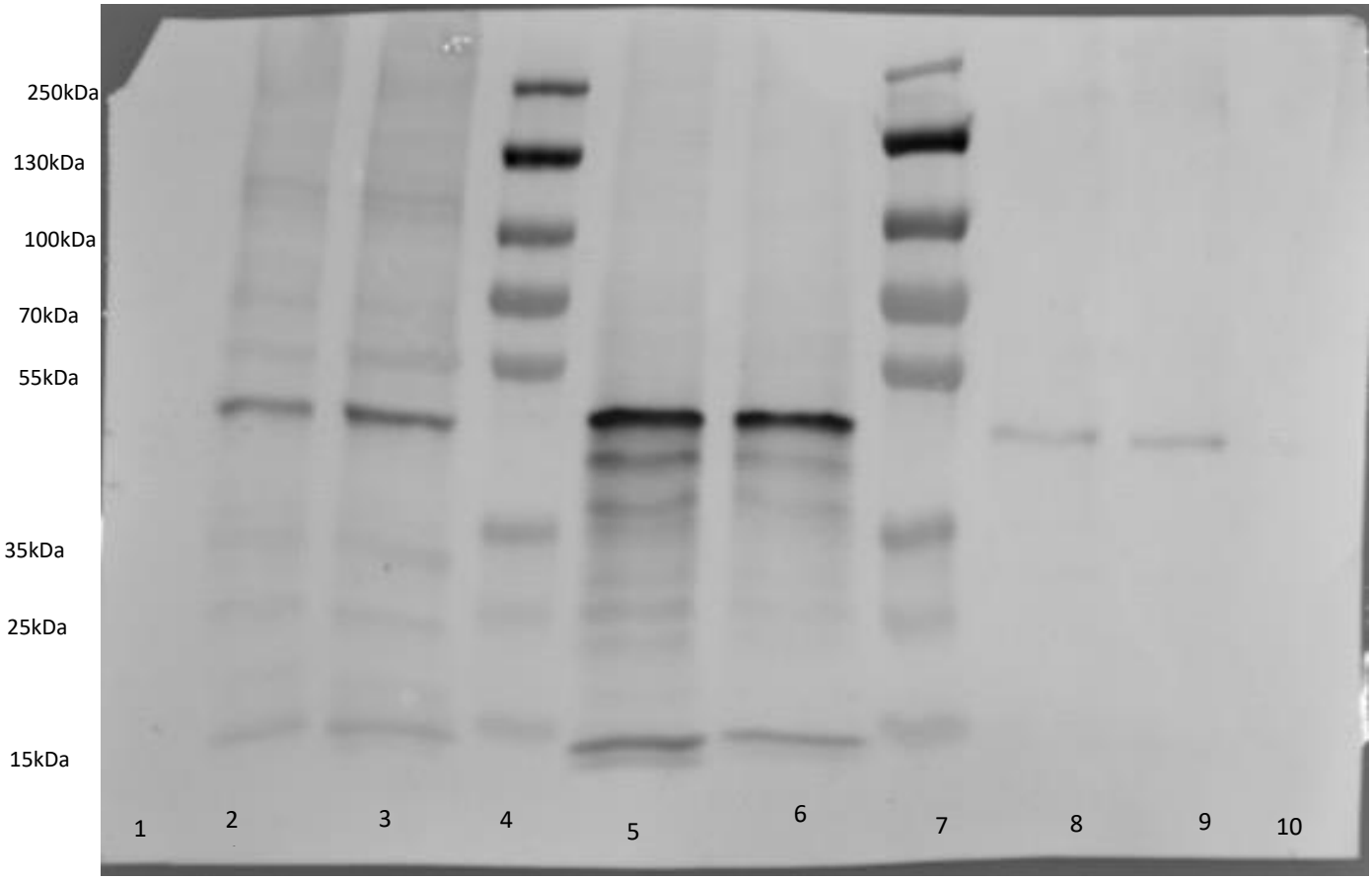


Figure 22; To investigate the presence of Flotillin-1 (1:1000) within the following samples; sEV derived from human brain tissue, cell homogenates and brain homogenates. Lanes 1 and 10 contained loading buffer as a negative control. Lane 2 fibroblast homogenate AD AG07872. Lane 3 fibroblast homogenate control AG08125. Lane 4 and 7 molecular weight markers. Lane 5; Human brain homogenate AD (15/08) Lane 6 Human brain homogenate control (15/30) Lane 8 Human brain sEV extract AD (15/08). Lane 9, Human brain sEV extract control (15/30).

The following lanes 2, 3, 5, 6, 8 & 9 contained a clear band just below 55kDa region which is an indication of flotillin-1 with a molecular weight of 47kDa. The positive controls in lanes 2 and 3 (whole cell homogenate) were a much lighter compared to the other positive controls (BHC) in lane 5 and 6. Lane 1 and 10 were negative controls and therefore, no bands were present as expected. The sEVs derived from human brain tissue contained a faint line in the correct region for flotillin-1 and can therefore say this is evidence to show that sEVs were successfully extracted from human brain tissue.

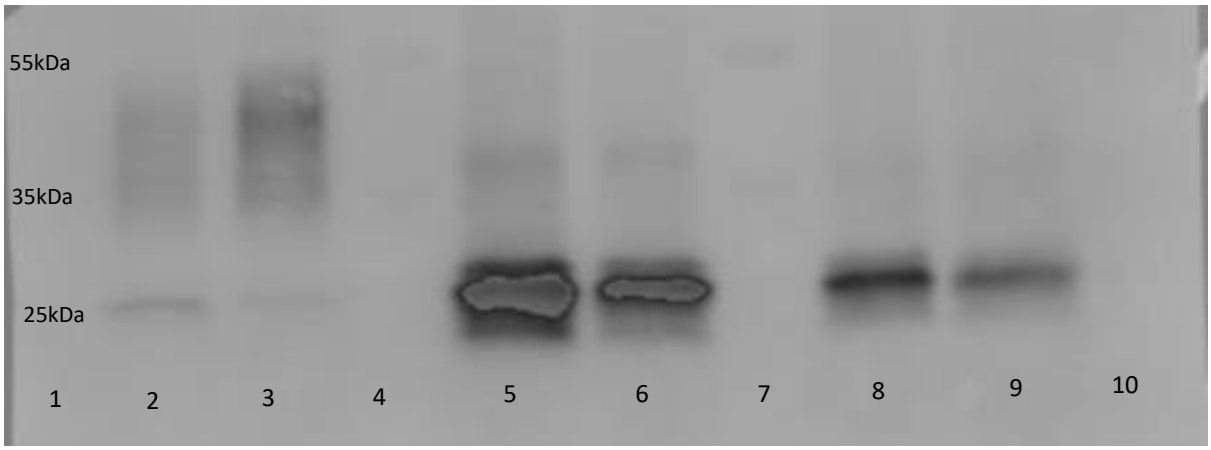


Figure 23; To investigate the presence of CD9 (1:1000) within the following samples; sEV derived from human brain tissue, cell homogenates and brain homogenate. Lanes 1 and 10 contained loading buffer as a negative control. Lane 2 fibroblast homogenate AD AG07872. Lane 3 fibroblast homogenate control AG08125. Lane 4 and 7 molecular weight markers. Lane 5; Human brain homogenate AD (15/08) Lane 6 Human brain homogenate control (15/30) Lane 8 Human brain sEV extract AD (15/08). Lane 9, Human brain sEV extract control (15/30).

Despite having a faded ladder due to this membrane being striped and re-probed for CD9, the following lanes 2, 3, 5, 6, 8 & 9 contained a clear band just below 25kDa region which is an indication of flotillin-1 with a molecular weight of 24kDa. The positive controls in lanes 2 and 3 (whole cell homogenate) were a much lighter compared to the other positive controls (BHC) in lane 5 and 6. Lane 1 and 10 were negative controls and therefore, no bands were present as expected. The sEVs derived from human brain tissue contained a faint line in the correct region for CD9 and can therefore is evidence to show that sEVs were successfully extracted from human brain tissue.

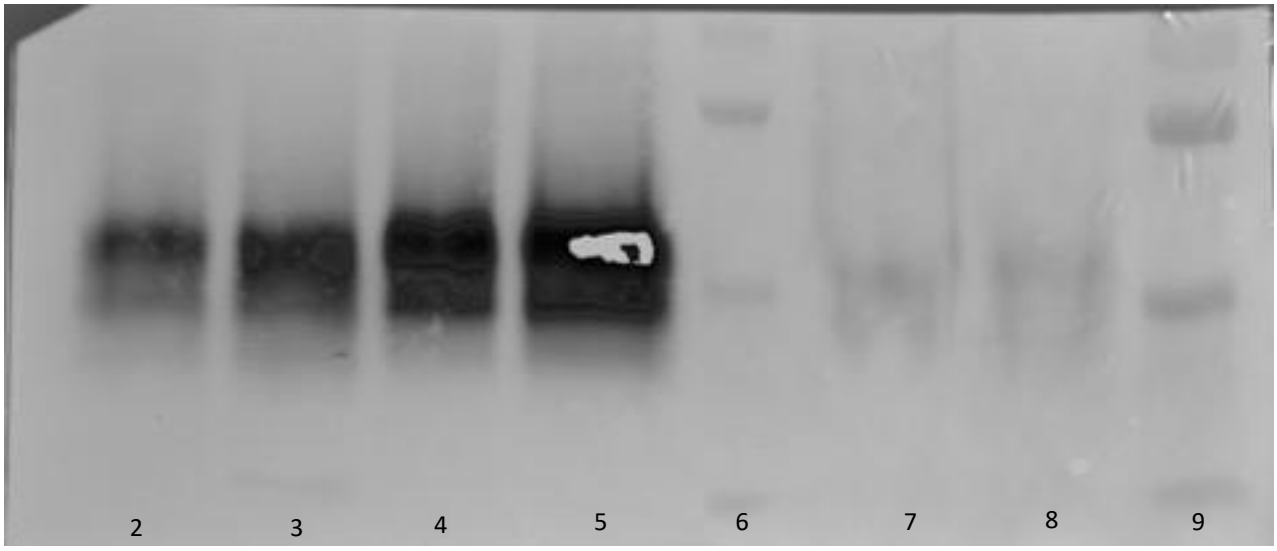


Figure 24; To investigate the presence of CD63 (1:2000) sEVs. Lanes 1 and 10 contained loading buffer as a negative control. Lane 2 Human brain homogenate control (15/01). Lane 3 Human brain homogenate AD (15/20). Lane 4 Human brain homogenate with collagenase control (15/01). Lane 5; Human brain homogenate with collagenase AD (15/20). Lane 6 and 9 molecular weight markers. Lane 7 Human brain sEV extract control (15/01). Lane 8, Human brain sEV extract AD (15/20).

Bands were present in the positive controls in lanes, 2, 3, 4 & 5 at the region of 35kDa despite, only being faint bands in the sEV samples in lane 7 & 8 there is a faint band at the 35kDa region. CD63 has a molecular weight of 35-55kDa and despite not being the best result visually, this result justifies that CD63 is present and sEVs have been successfully extracted from human brain samples.

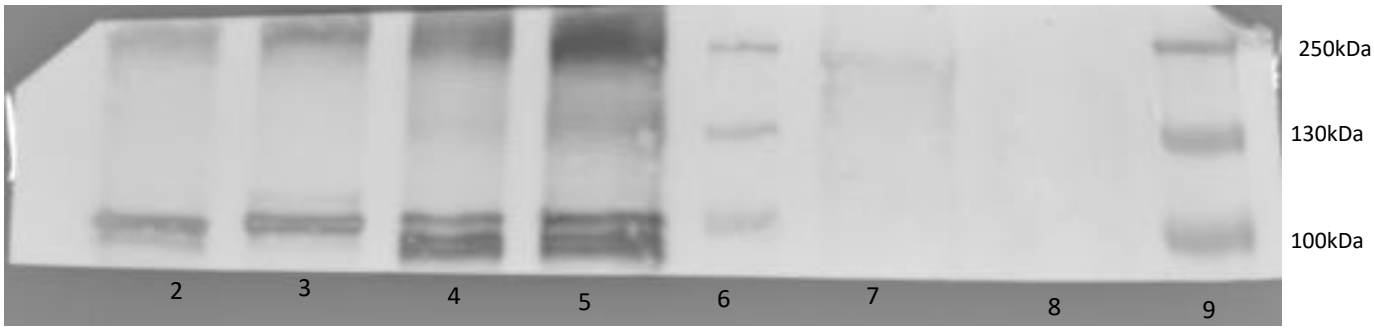


Figure 25: To investigate the presence of Calnexin (1:1000) sEVs. Lanes 1 and 10 contained loading buffer as a negative control. Lane 2 Human brain homogenate control (15/01). Lane 3 Human brain homogenate AD (15/20). Lane 4 Human brain homogenate with collagenase control (15/01). Lane 5; Human brain homogenate with collagenase AD (15/20). Lane 6 and 9 molecular weight markers. Lane 7 Human brain sEV extract control (15/01). Lane 8, Human brain sEV extract AD (15/20).

Bands were present in the positive controls in lanes, 2, 3, 4 & 5 below the of 100kDa and is therefore proof of calnexin being present within the positive controls which is expected below the 100kDa due to having a molecular weight of 90kDa. There is a band present at the 250kDa in lane 7 but this is not in the correct region for calnexin and is therefore, non-specific binding. Calnexin should be absent in the sEV samples and is used as a negative marker. This is evidence that a pure population of sEVs have been extracted from human brain samples.

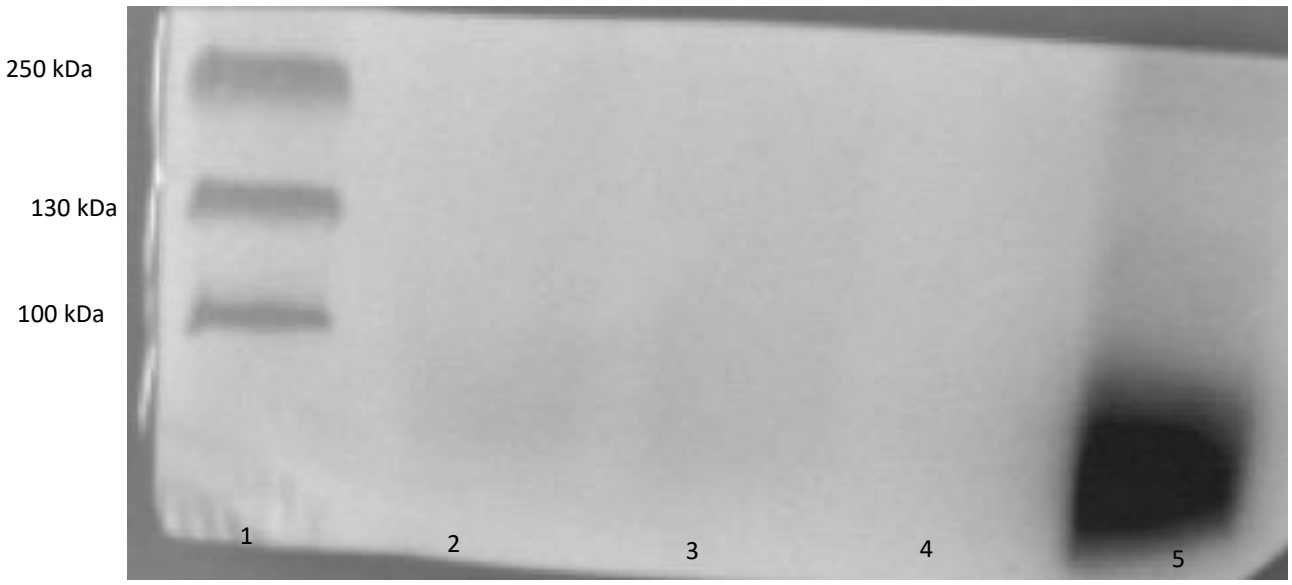


Figure 26; To investigate the presence of calnexin (1:1000) in human brain derived sEV samples. Lane 1; the molecular weight protein ladder. Lane 2; Human brain sEV extract AD (15/21). Lane 3; Human brain sEV extract control (15/31). Lane 4; was empty and lane 5; Human brain homogenate with collagenase control (15.31).

The membrane was cut just above 70kDa. There were no bands present within lanes 2 and 3 however, there was a band present in lane 5 below the 100kDa region. This is an indication of calnexin, as calnexin has a molecular weight of 90kDa which is seen in the positive control in lane 5. Calnexin is not expected to be present in sEV samples therefore, this is clear evidence that sEVs were successfully extracted from human brain tissue.

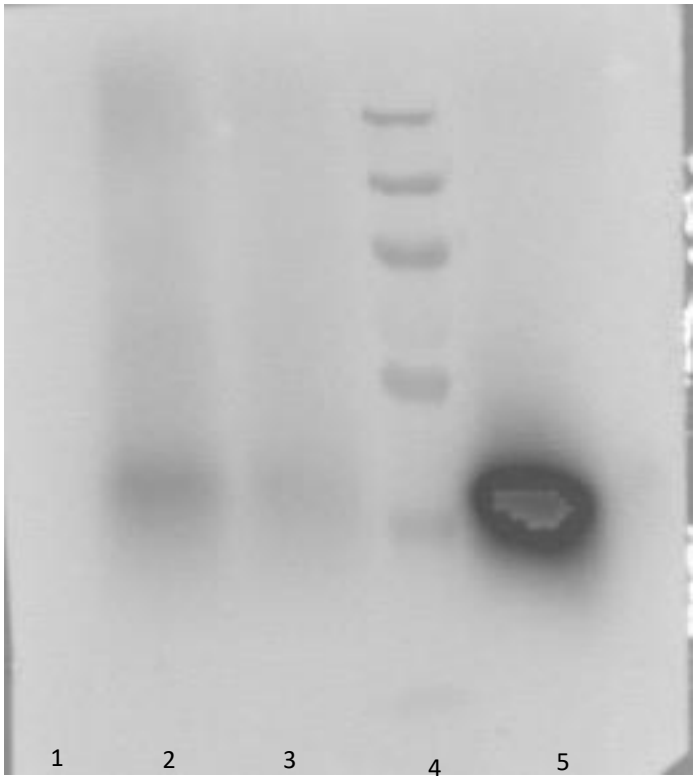


Figure 27; To investigate the presence of CD63 (1:2000) human derived sEVs. Lane 1; is an empty lane. Lane 2 Human brain sEV extract control (15/31). Lane 3; Human brain sEV extract AD (15/21). Lane 4 is the molecular weight protein ladder and lane 5; Human brain homogenate with collagenase control (15.31).

In the sEV sample lanes 2 and 3 there are clear bands present at the 35kDa region. CD63 has a molecular weight between 35kDa and 55kDa. The positive control in lane 5 has a clear band present therefore, this is a clear indication of CD63 and can therefore say sEVs were successfully extracted from human brain tissue.

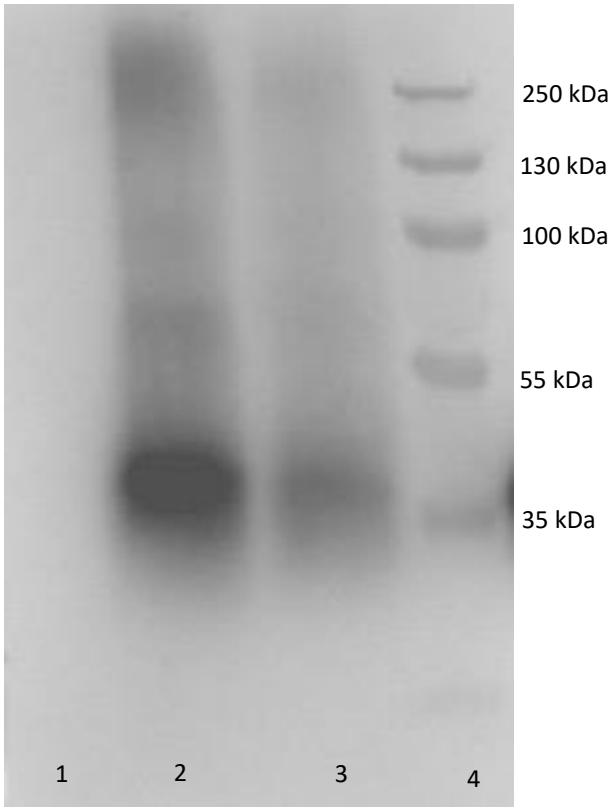


Figure 28; To investigate the presence of CD63 (1:2000) in human brain derived sEVs without the positive control. Lane 1; Empty lane. Lane 2 Human brain sEV extract control (15/31). Lane 3; Human brain sEV extract AD (15/21). Lane 4 is the molecular weight protein ladder.

This membrane is the same as the figure above without the positive control present in lane 5, this membrane was cut to overexpose the sEV lanes to get a better image of the bands present at 35kDa indicating CD63.

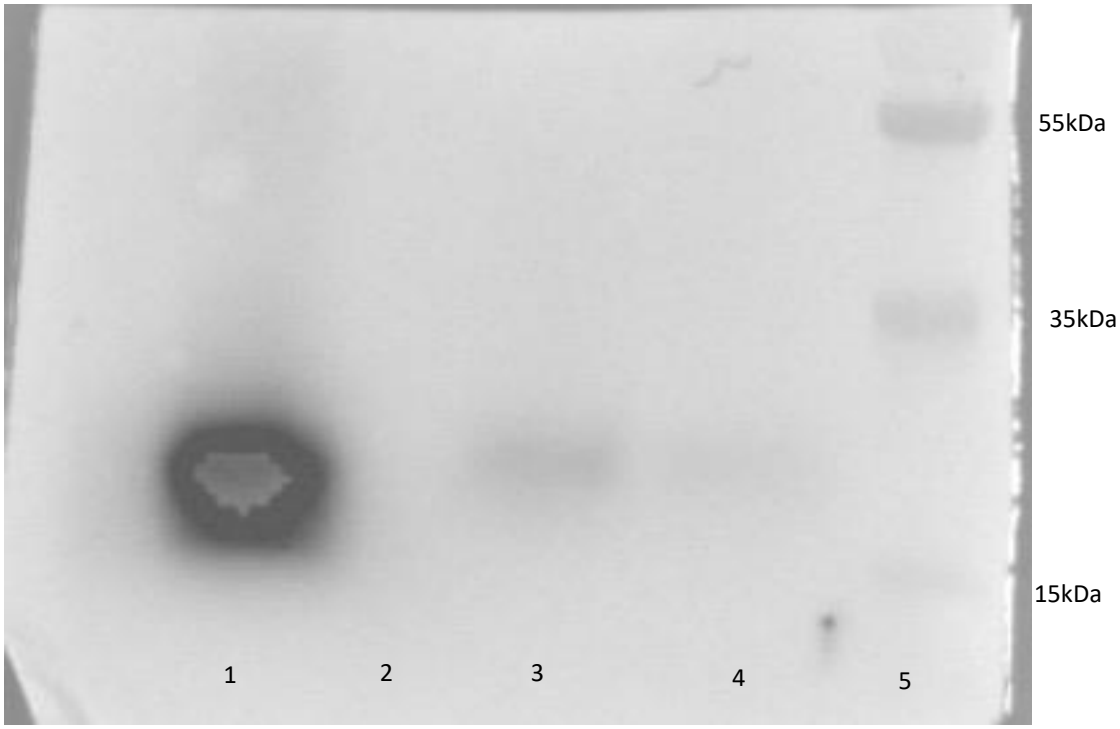


Figure 29; To investigate the presence of CD9 (1:1000) in human brain derived EVs. Lane 1; Human brain homogenate with collagenase control (15.31). Lane 2; Empty lane. Lane 3; Human brain sEV extract control (15/31). Lane 4; Human brain sEV extract AD (15/21). Lane 5: molecular weight ladder.

There is a distinct band in lane 1 which is the positive control and 2 faint bands in lane 3 and in lane 4 which are the sEV derived samples. The bands are located above 15kDa but below 35kDa, despite seeing it clearly on the molecular weight ladder the bands are present at the 25kDa region. CD9 has a molecular weight of 24kDa therefore, this is a clear indication that CD9 is present within the sEV samples as well as in the positive control. This is evidence to show that sEV were successfully extracted from human brain tissue.

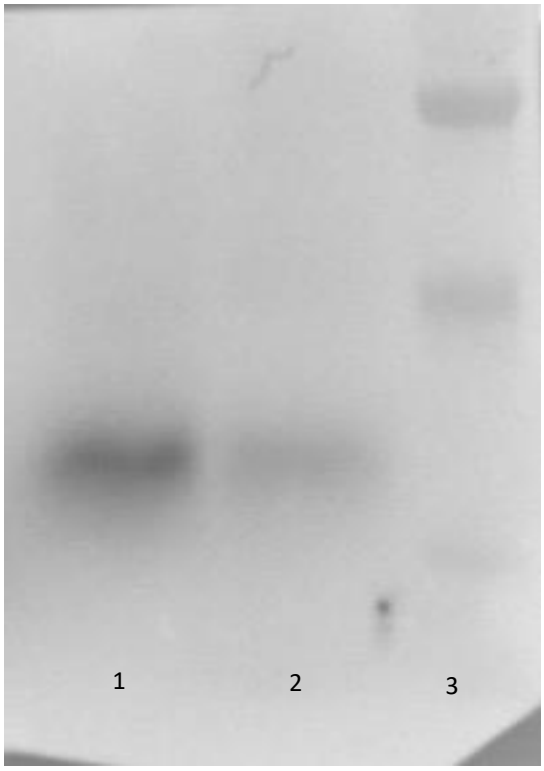


Figure 30; To investigate the presence of CD9 (1:1000) in human brain derived EVs without the positive control. Lane 1; Human brain sEV extract control (15/31). Lane 2; Human brain sEV extract AD (15/21). Lane 3: molecular weight ladder.

This figure is the same figure as above without the presence of the positive control in lane, the membrane was cut between the positive control and the sEV lane this was to allow the sEV lanes to be overexposed so the bands within the sEV lanes were exposed and were able to get a clearer image of the bands. The image shows 2 bands present within the region of 25kDa therefore, this is evidence to show that sEV were successfully extracted from human brain tissue.

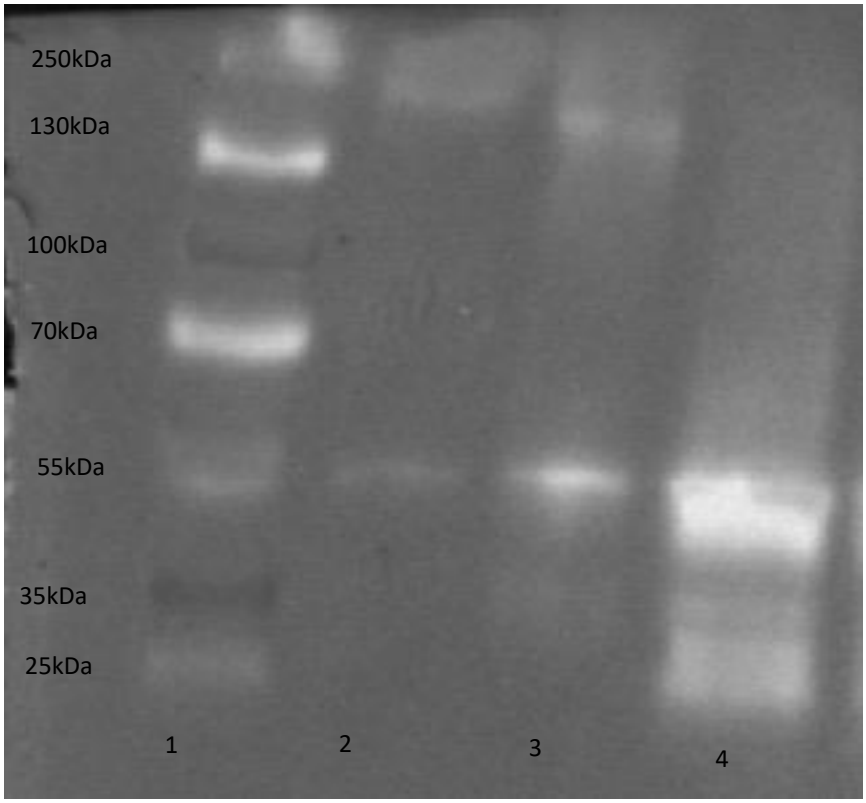


Figure 31; To investigate the presence of flotillin-1 (1:1000) within sEV samples. Lane 1; Molecular weight marker. Lane 2; Human brain sEV extract AD (15/08). Lane 3; Human brain sEV extract control (15/31). Lane 4; Human brain sEV extract AD (15/21). Lane 5: Human brain homogenate control (15.31).

Bands were present in lanes, 2, 3 and 4 at the 55kDa region, flotillin-1 has a molecular weight of 47kDa. The positive control in lane 4 shows the presence of a band within the correct region therefore, this is a clear indication of Flotillin-1. This evidence shows that sEVs were successfully isolated from human brain tissue.

In the human brain tissue samples, the EV markers CD9, CD63 and Flotillin-1 were highly expressed in majority of experiments despite, one where the bands were faint for CD63 see figure 24. The calnexin marker (an endoplasmic reticulum marker) was not expressed in the sEV sample but was expressed in the whole brain homogenates, brain homogenates with collagenase and cell lysates. The results from these experiments match the MISEV2018 guidelines by identifying three sEV specific markers in isolates and can therefore, successfully show that sEVs were extracted from human brain tissue.

3.4.2 Human Brain characterisation – NTA

Nano-tracking analysis was another method recently developed in the group and so to minimise waste of the precious human materials, this method was used in the first instance to confirm the presence of sEVs through detection of particles of the EV size range of <200nm. NTA using the random movement of particles called the "Brownian motion" to measure the number of particles and the particle size. NTA also has a fluorescent setting known as fNTA which allows the sample to be mixed with sEV associated antibodies to visually confirm the presence of sEV within samples prior to this the cell mask orange stain is used as a quantitative method to determine the number of biological particles from the non-biological particles. The CMO stain works by staining the plasma membrane of biological particles and has an excitation peak of 556nm. This analysis was done on 3 AD patient derived samples and 3 healthy aged control samples with three technical repeats, to investigate the presence and absence of sEVs using sEV specific antibodies. Samples were not age matched within experiments and were carried out on based on the amount of brain tissue that was available at the time.

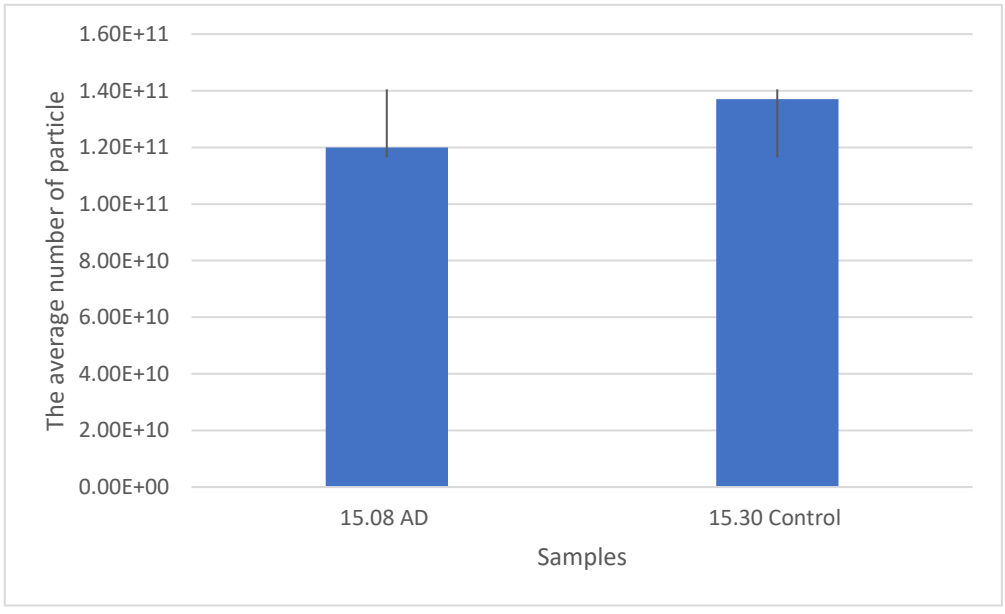
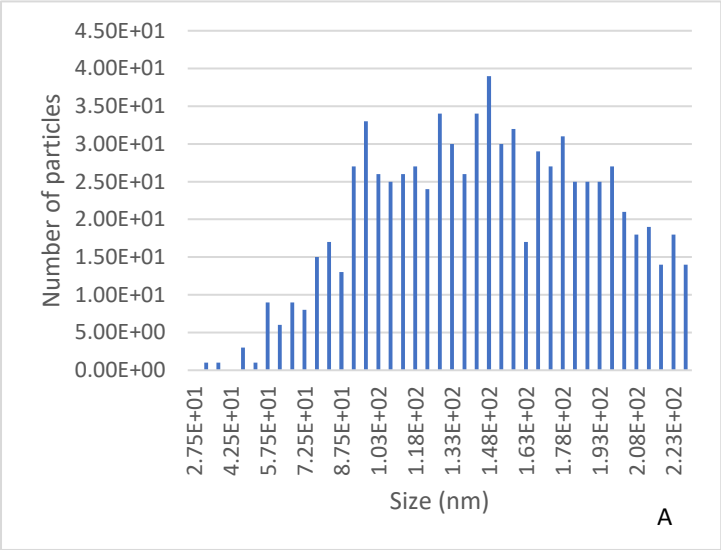


Figure 32: To investigate the average number of particles from samples 15.08 and 15.30 with a size range between 2.5nm and 6025nm.

The total number of particles are higher in the controlled sample (15.30) compared to the AD sample (15.08), the control sample contained 1.37E+11 particles whereas the AD sample contained 1.2E+11 particles.



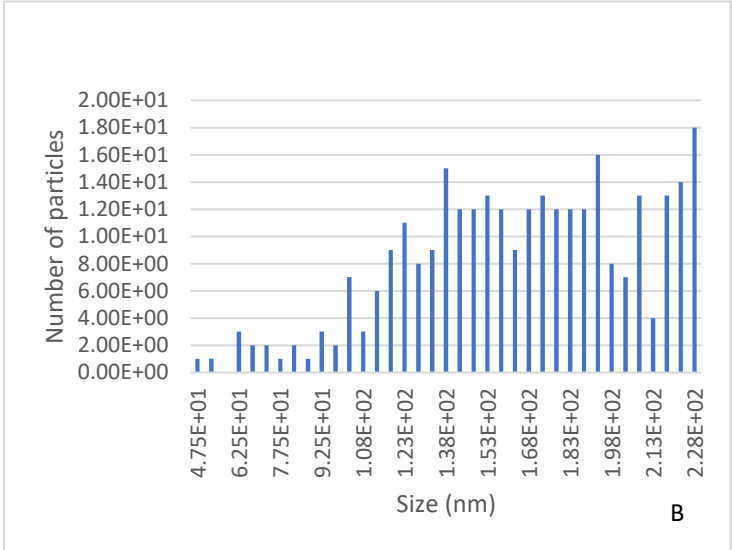


Figure 33; fNTA experiment to investigate the number of biological particles present within samples 15.08 (A) and 15.30 (B) within the range of 2.75nm and 228nm.

Graph A is the AD sample with an average particle size of 190.1nm which is within the sEV range whereas the average sized particles within graph B the controlled sample is lightly higher at 221.6nm which is not within the sEV range, particles need to be below <200nm for it to be considered to be within the sEV size range. The curve in graph B is slightly more to the right indicating the particles within this sample are bigger particles. Despite having biological particles within the size range of sEV, none of the CD markers for these samples shown up and therefore this shows the methodology may need optimising thoroughly to get a positive result for the antibody markers.

In the first instance, manufacturing recommendations were followed when completing the fNTA experiments for the antibodies however, the incubation period stated a minimum of an hour which could be the possible reason for the CD markers not working in the first instance therefore, for further experiments regarding the CD markers the incubation period will be for at least 2 hrs.

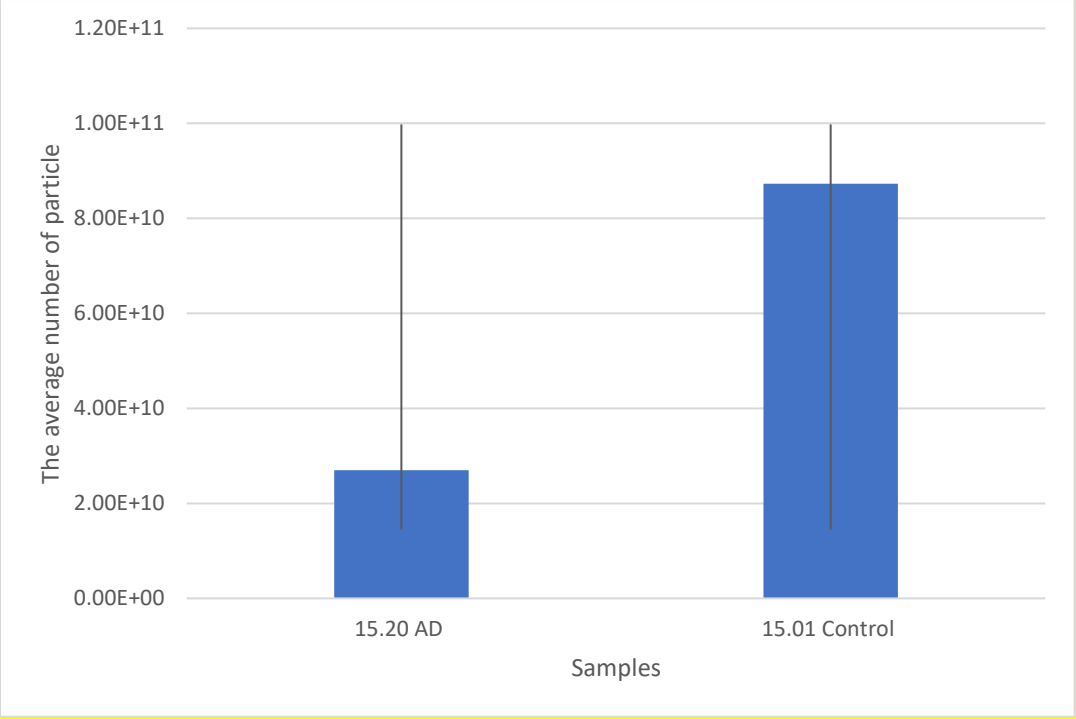


Figure 34; To investigate the total number of particles present between the size range of 2.5nm - 6025nm in both the control sample 15.01 and Alzheimer's Disease sample; 15.20

The total number of particles present within the AD sample 15.20 and the controlled sample 15.01, the control sample contains over double the number of particles compared to the AD sample, which is the opposite of previous scatter experiment. AD sample contains 2.7E+10 particles whereas the controlled sample contains 8.73E+10 particles.

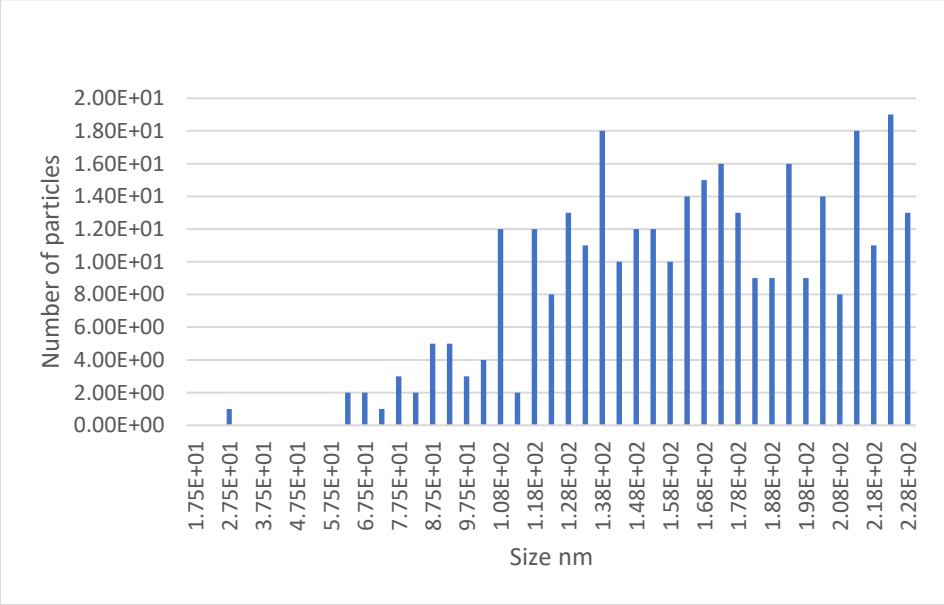


Figure 35; To investigate the number of biological particles in the Alzheimer's Disease sample 15.20 and the size range of the particles present between 2.75-228nm in size.

The size range of the biological particles is between 27.5nm and 228nm however, the vast majority of particles start at the size range 128nm. The total number of biological particles obtained from this sample is very low, the highest number peak contains 19 particles but is outside of the sEV range with a size of 223nm.

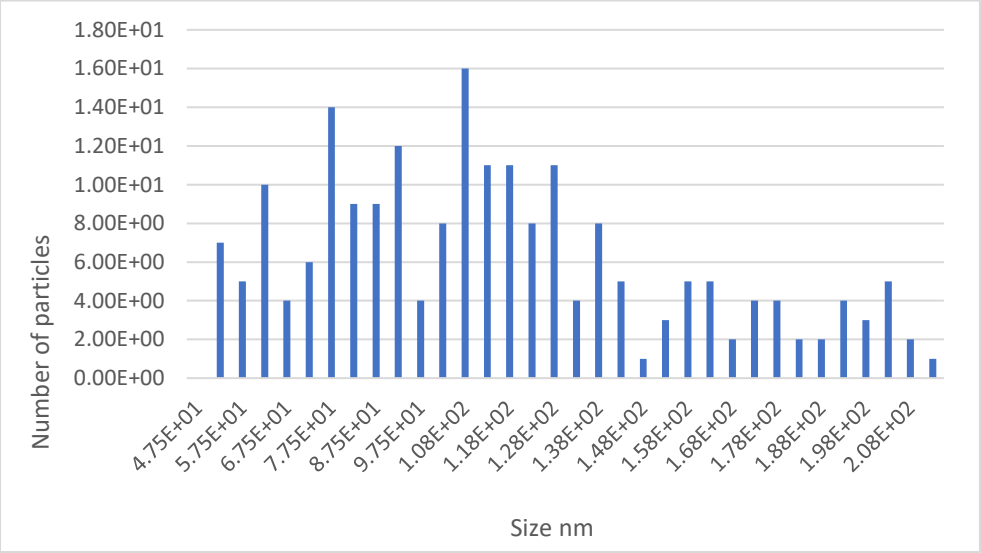


Figure 36; To investigate of particles containing CD9 within the controlled sample 15.01 within the range of 47.5nm - 213nm.

This experiment shows that sEV are present within this sample as multiple particles have been tagged with the sEV marker CD9, the majority of the particles are within the sEV size range of <200nm and is therefore, possible to say this sample shows that sEVs are present and were successfully isolated from human brain tissue.

In the first instance, the AD case 15.20 had no traces of CD9, CD63 or CD81 within the sample but did contain biological particles within the EV range according to the CMO experiment and the sample 15.01 had no traces of CD63 or CD81, despite containing traces of CD9 within the sample. Due to lack of positive results in this time round, despite having to stick to the final antibody dilution of 1:100,000 for the CD markers to preserve reagents, the sample dilution and the dye dilutions can be altered to try and optimise the number of particles detected within the sample.

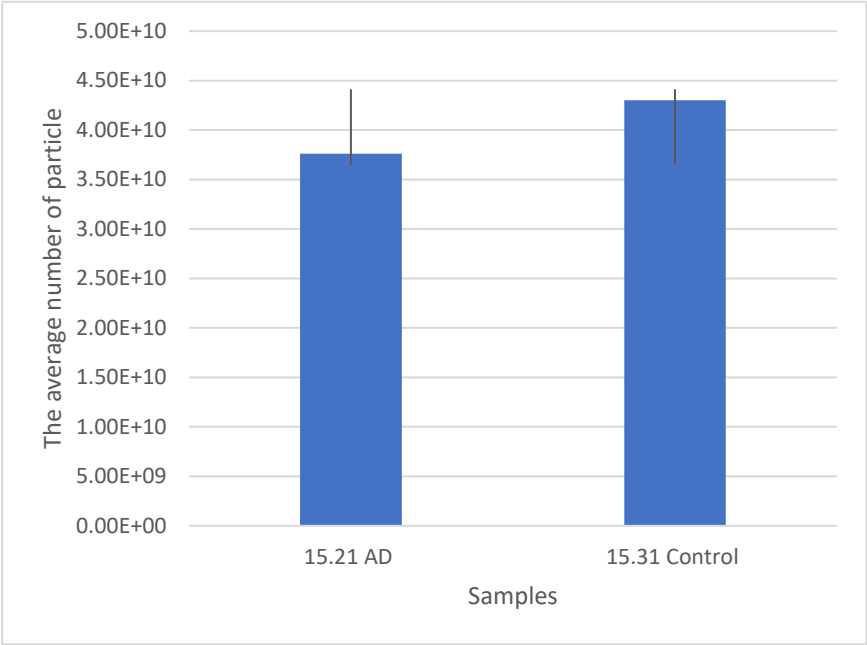


Figure 37; To investigate the total number of particles present between the size range of 2.5nm - 6025nm in both the control sample 15.31 and Alzheimer's Disease sample; 15.21

The control sample contains more particles than the AD sample, the average number of particles within the controlled sample is 4.3E+10 particles whereas the AD sample contains 3.76E+10 particles, this data shows that there are significantly more particles present within the control sample compared to the AD sample.

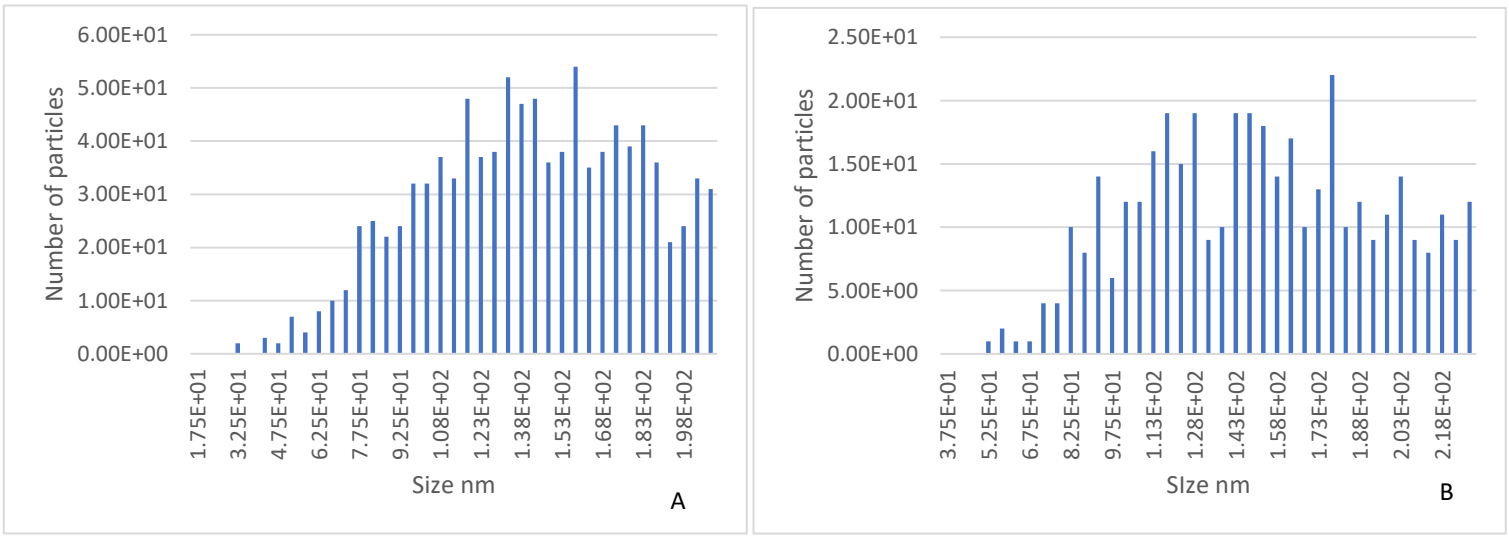


Figure 38; To investigate the number of biological particles in the AD sample 15.21 (A) and the controlled sample 15.31 (B) and the size range of the particles present between 17.5-218nm.

In both the graphs it shows there are biological particles present within the sEV range of <200nm however, in graph A it does show to have a much higher number of particles with the same size range compared to those in graph B. The highest peak in graph A is at 158nm with a number of 55 particles at that particular size however, the highest peak in graph B is 22 particles at 178nm in size. Both of which are within the sEV range however it does show that graph A contains more particles and the shapes of the graphs do vary due to lack of particles within graph B. Despite this, both of the graphs main population are within the middle of the graph within the sEV size range of sEVs of <200nm. The average size of particles in the AD sample 15.21 is 180.6nm however, the average sized particles within sample control sample 15.31 are 193.7nm. This data does suggest that the particles within the control sample 15.31 are much bigger compared to those within the AD sample.

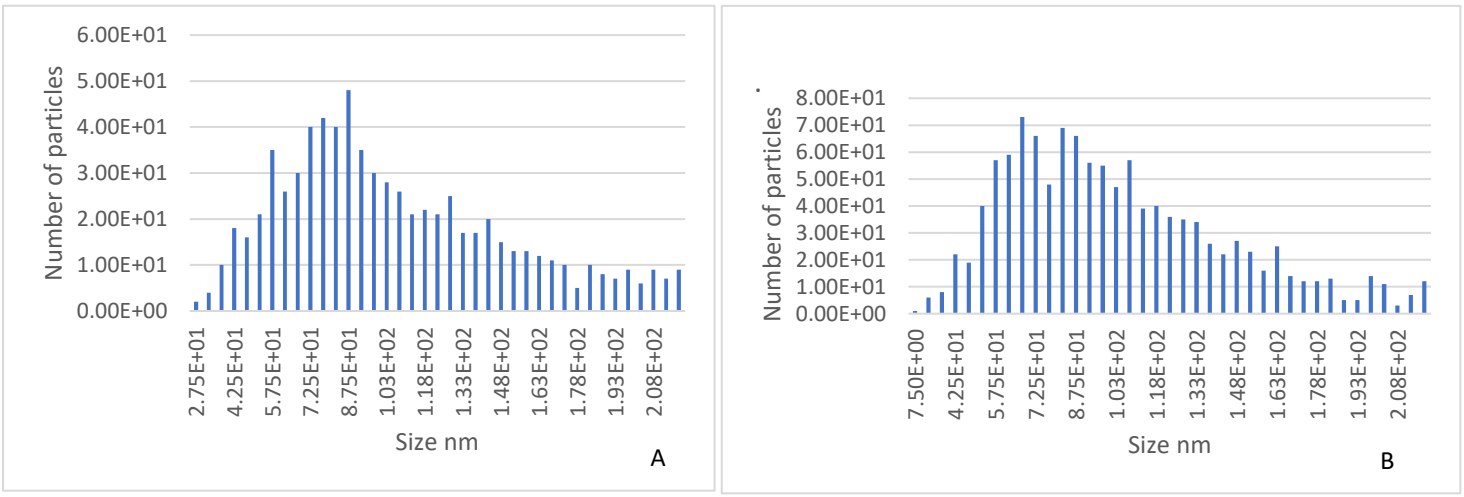


Figure 39; To investigate the number of particles tagged with CD9 antibody present within samples 15.21 (A) and 15.31 (B) within the range of 2.75nm and 223nm.

In graph B it shows the number of particles with the same size is much higher compared to graph A. The shape of the curve within both graphs is very similar and is shifted more to the left indicating much smaller particles present. The average sized particles in graph A are 122.8nm whereas the averaged size particles in graph B are 118.6nm showing that the particles within graph B are slightly smaller on average compared to those in graph A. The graphs show the present of CD9 within both samples and therefore, this is evidence that sEVs have been extracted from these 2 samples.

Not all the samples contained the present of sEVs however, by carrying out optimisation steps the particles detected increased. The present of CD81 in all the samples were 0 and therefore, this could be researched further. However, this method shows that sEVs can be detected using fNTA.

Table 11: To present the average number of particles and the average size of particles tagged with CD9 antibody.

Sample	Average number of particles.	Average size of biological particles (nm)	Average size of particles tagged with CD9 (nm).
15.20	2.70E+10	277.4	444.8
15.21	3.76E+10	180.6	122.8
15.08	1.20E+11	190.1	252.5
15.01	8.73E+10	282.7	202.4
15.31	4.3E+10	193.7	118.6
15.30	1.37E+11	221.6	183.6

The samples which contained very little particles and therefore, the average size particles are much bigger and not within the sEV range. The samples which obtained a good number of particles and shown a positive reading for CD9 (AD case 15.21 and controlled case 15.31) shown a much more expected result which is within the sEV range. Controlled sample 15.30 also had an averaged size result which is within the sEV range of <200nm. Due to not all samples obtaining an CD63 result, it would make the result inaccurate as only 1 positive CD63 result came from the controlled sample and it would be meaningless to compare against 3, CD63 results obtained from the AD samples only, if there was more time available this would have been researched further. The AD samples show to have an average particle size within the sEV range compared to the controlled samples, the average sized particles within the AD samples seem to be much bigger however, this may be due to the AD samples containing more particles and therefore further tests would need to be done to confirm whether this is consistent across disease groups or just within these experiments.

For the sake of statistical testing 3 of each disease group was analysed using an unpaired t-test, to compare any variation within the disease groups. The following samples were

included; 3 x AD; 15.20, 15.21, 15.08 and 3 x control; 15.01, 15.31 and 15.30. The possible variations were tested; the number of particles within disease groups and the size of the particles present within the sEV sample using CD markers which are shown below.

The P value from the unpaired t-test, to test the potential variation in relation to the number of particles present within the AD sample and the control was 0.53 and is therefore, insignificant, anything greater than 0.05 is insignificant and means there is no variation within the groups in relation to the number of particles. The size of the particles within the AD and control sample were compared to test for any variation, with a P value of 0.38 means it is insignificant and there is no variation between the disease groups in regard to the size of the particles within the disease groups.

These results are further evidence for the successful isolation of sEVs from human brain tissue.

3.5 Western Blots; AT8, P62 and Beta Actin

Western blots were used to probe for the following protein markers; AT8 (Hyperphosphorylated tau) & P62 (an initial autophagy/protein aggregation marker). The antibodies were used to confirm the presence of the AT8 and P62 within the whole brain homogenate with collagenase but also in the extracellular vesicle fractions. Beta actin was used as a loading control, to prevent overload of protein content in one or more of the wells. These experiments were to investigation the cargo of AT8 and P62 within the BHC and sEVs across different braak groups; from lower braak groups (controlled) to higher braak groups with an AD diagnosis. The protein load of BHC samples were 20µg/µl and the protein load of sEV samples were 50µg/µl.

3.5.1 Whole brain homogenate and sEV - AT8

The antibody AT8, symbolises the present of hyperphosphorylated tau within a sample. Six human sample were used; 3 in a lower braak group and 3 in the higher braak group with a

diagnosis of AD. The BHC and sEV samples were both probed for AT8, in hope to see the protein cargo changes as the braak group increases.

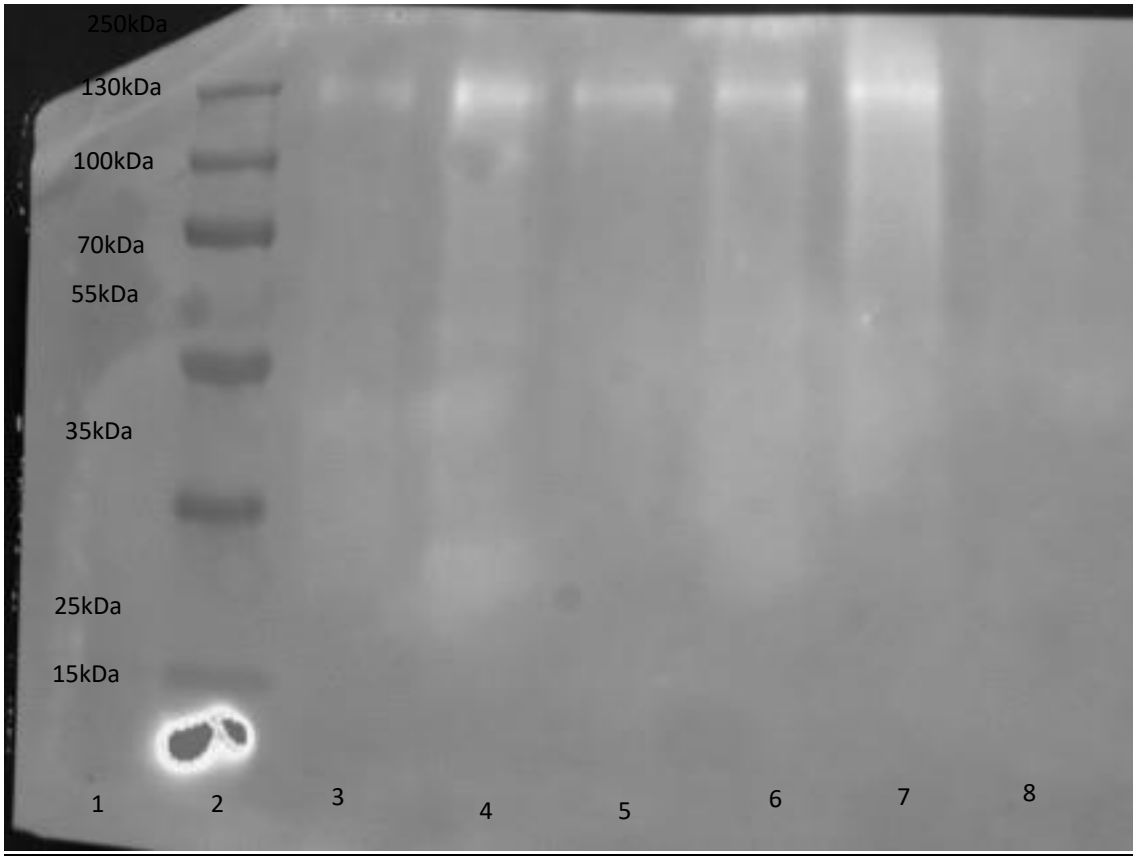


Figure 40; To investigate the presence of AT8 in the whole brain homogenate with collagenase lane 1 - Blank. Lane 2 contained the protein molecular weight ladder. Lane 3; Human brain homogenate control (15/26). Lane 4 Human brain homogenate control (15/31). Lane 5 Human brain homogenate control (15/30). The last 3 lanes were patient samples which were in the higher braak group with a diagnosis of AD. Lane 6 Human brain homogenate AD (17.07), Lane 7 Human brain homogenate AD (15.08), Lane 8 Human brain homogenate AD (15.20).

Bands were present in lanes 3-7 at the top of the membrane at the 250kDa region, this is not indication of AT8 being present. To be able to justify the presence of AT8, there would need to be a band at 60kDa. There were no bands presence in lane 1 and 8. No positive controls or negative as AT8 was expected to show in all brain homogenates to some degree.

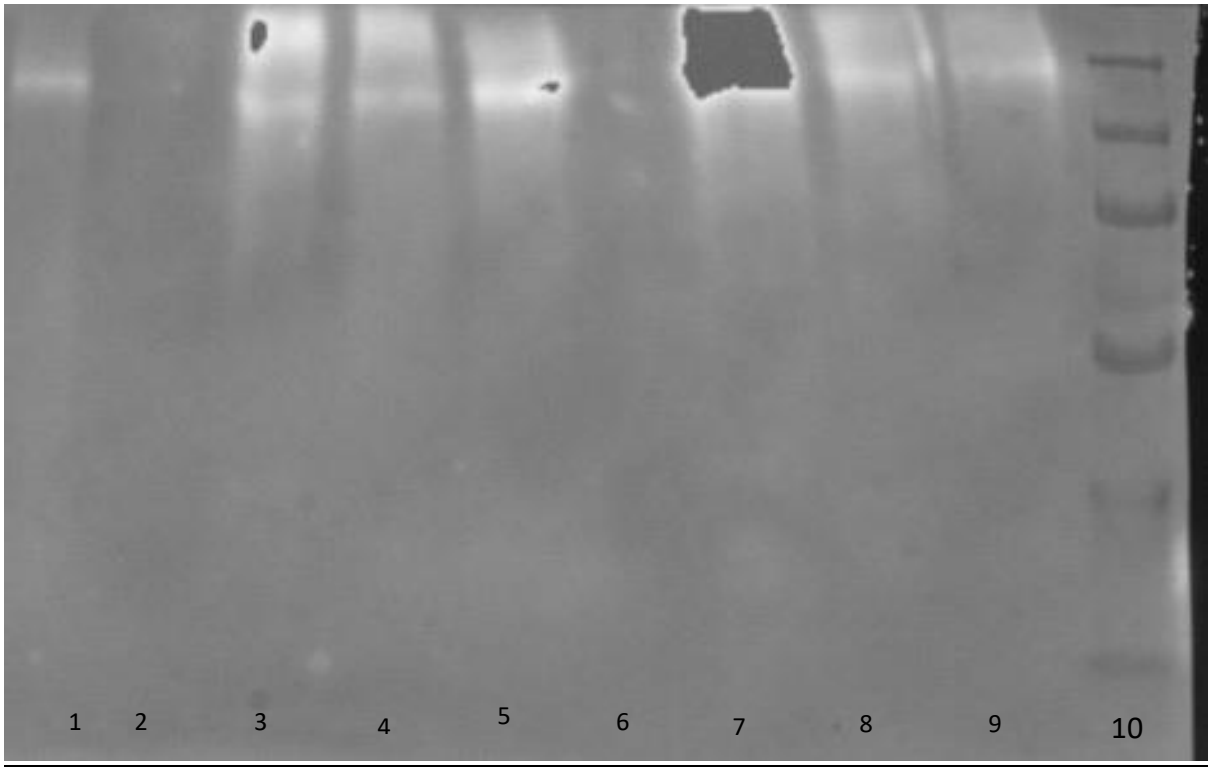


Figure 41; To investigate the presence of AT8 in the sEVs. Lane 1 positive control using brain homogenate with collagenase from sample 15.31. Lane 2 was a gap lane. Lane 3 Human brain sEV extract AD (15.20). Lane 4 Human brain sEV extract AD (15.08). Lane 5 Human brain sEV extract AD (17.07). Lane 6; Gap. Lane 7 Human brain sEV extract control (15/30). Lane 8 Human brain sEV extract control (15/31). Lane 9; Human brain sEV extract control (15/26). Lane 10 molecular weight ladder.

The bands in lanes 1, 3,4,5, 7, 8 and 9 expressed at the top of the wells at region 250kDa therefore, this is not a clear indication of AT8 being present within the sample as AT8 is expected at the 60kDa region of the molecular weight ladder. AT8 was not expressed in either of the samples, further antibody optimisation may be needed in order to identify AT8 clearly.

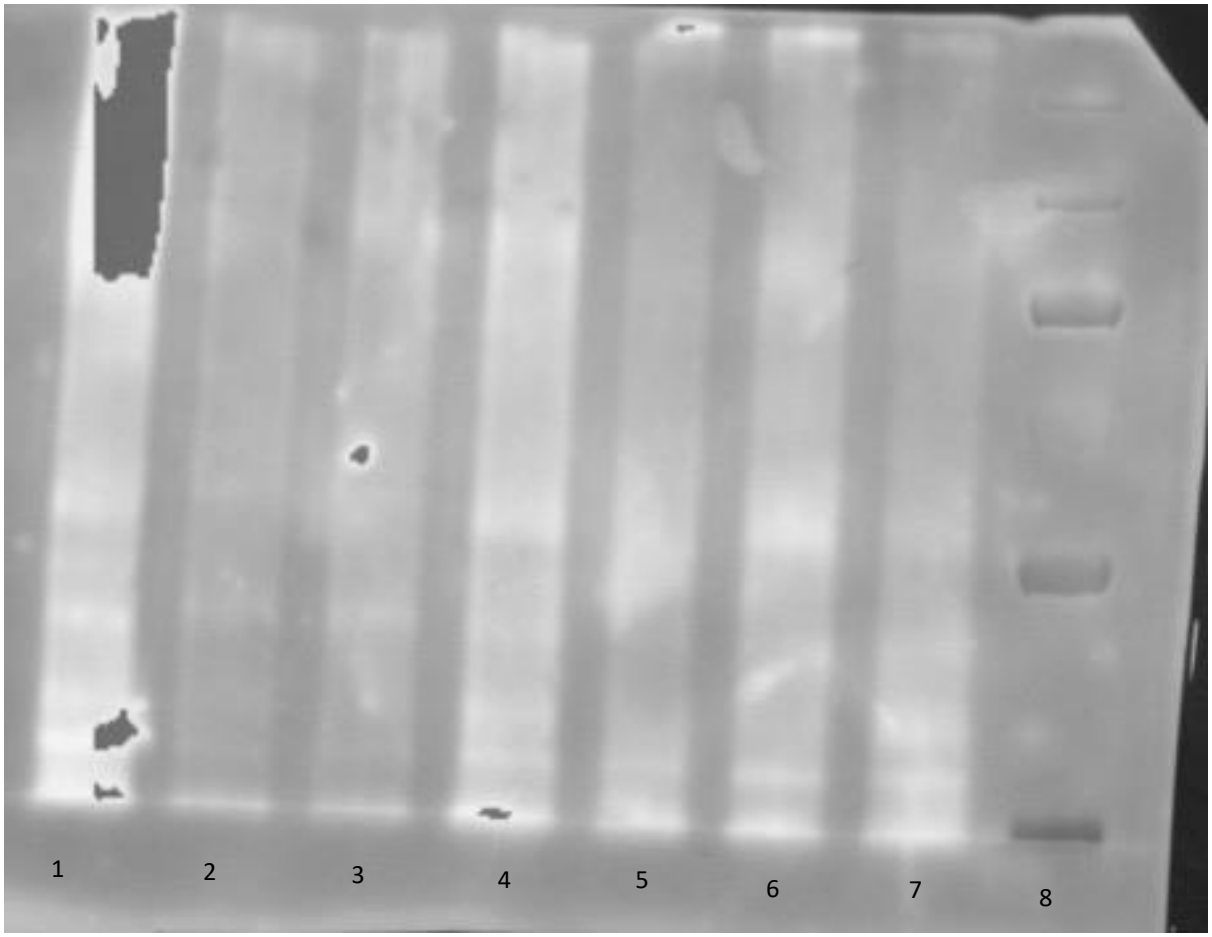


Figure 42: To investigate the presence of AT8 in the whole brain homogenate samples.
Lane 1 Human brain homogenate AD (15.20). lane 2 and 3 both Human brain homogenate AD (15.08) (human error). Lane 4 Human brain homogenate AD (17.07) Lane 5 Human brain homogenate control (15.30). Lane 6 brain homogenate control (15.31). Lane 7 brain homogenate control (15.26). Lane 8 protein molecular weight ladder.

This membrane is very a messy blot therefore, not of the bands are seen clearly. However, there is a lot of non-specific binding evident. A clear band at the region of 60kDa would have been a clear indication of AT8 being present within the lanes and therefore, from this result AT8 was not confirmed in the samples.

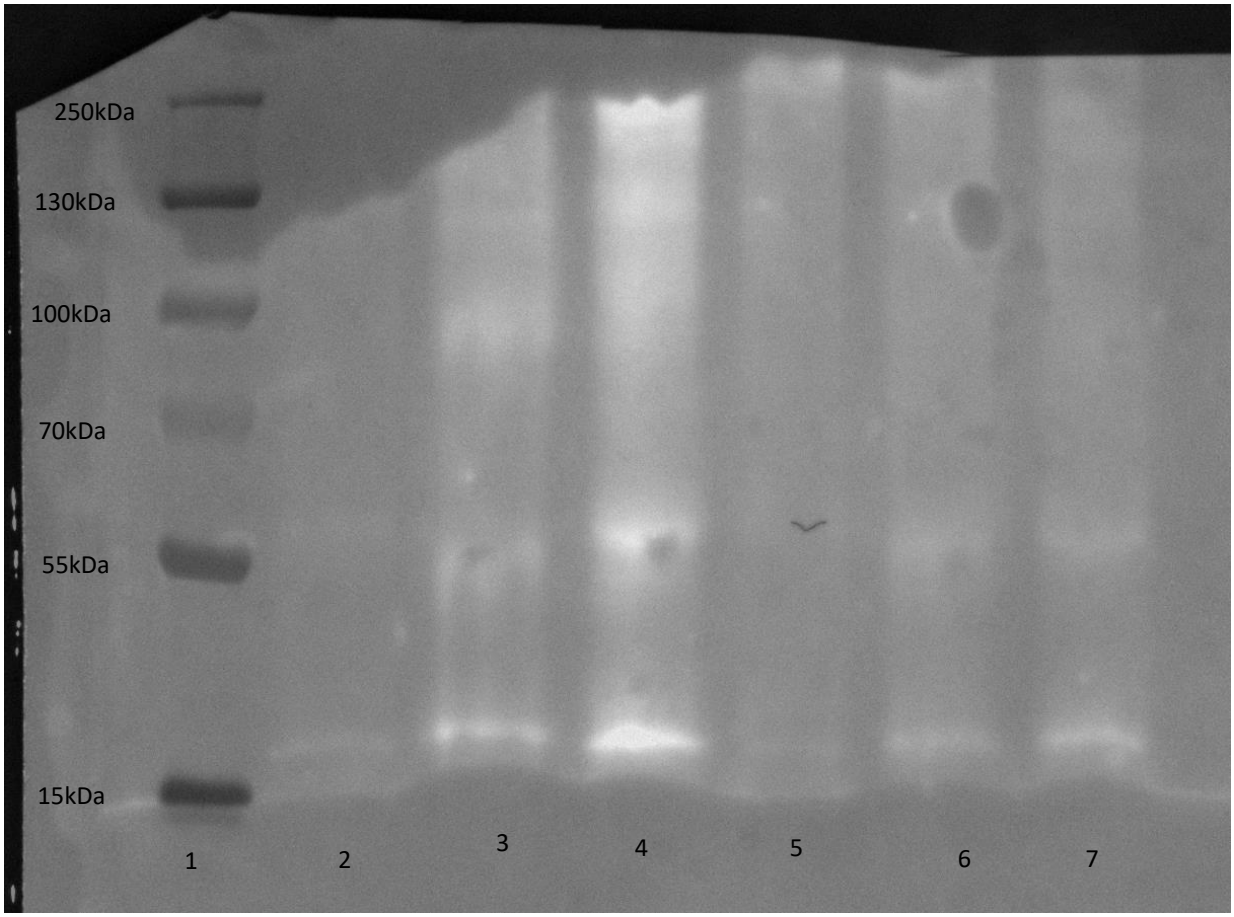


Figure 43; To investigate the presence of AT8 within the sEV fractions. Lane 1; protein molecular weight ladder. Lane 2; Human brain sEV extract control (15/26). Lane 3 Human brain sEV extract control (15/31). Lane 4 Human brain sEV extract control (15/30). Lane 5 Human brain sEV extract AD (17.07). Lane 6 Human brain sEV extract AD (15.08). Lane7: Human brain sEV extract AD (15.20).

In lanes 3, 4, 6 & 7 there is a faint band at the 55kDa region the band is not present in lane 2 or lane 5. AT8 is expressed around the 60kDa mark therefore, this could potentially be an indication of AT8 being present in the sEV fractions. However, there was no positive control used in this membrane and in the AT8 experiments prior to this, AT8 did not show up with any bands within the correct region.

3.5.2 Whole brain homogenate and sEV - P62

Western blots probed for p62 an initial autophagy marker, p62 was probed for in BHC and sEVs of 6 samples, 3 of which had a diagnosis of AD and 3 which were controlled patient samples.

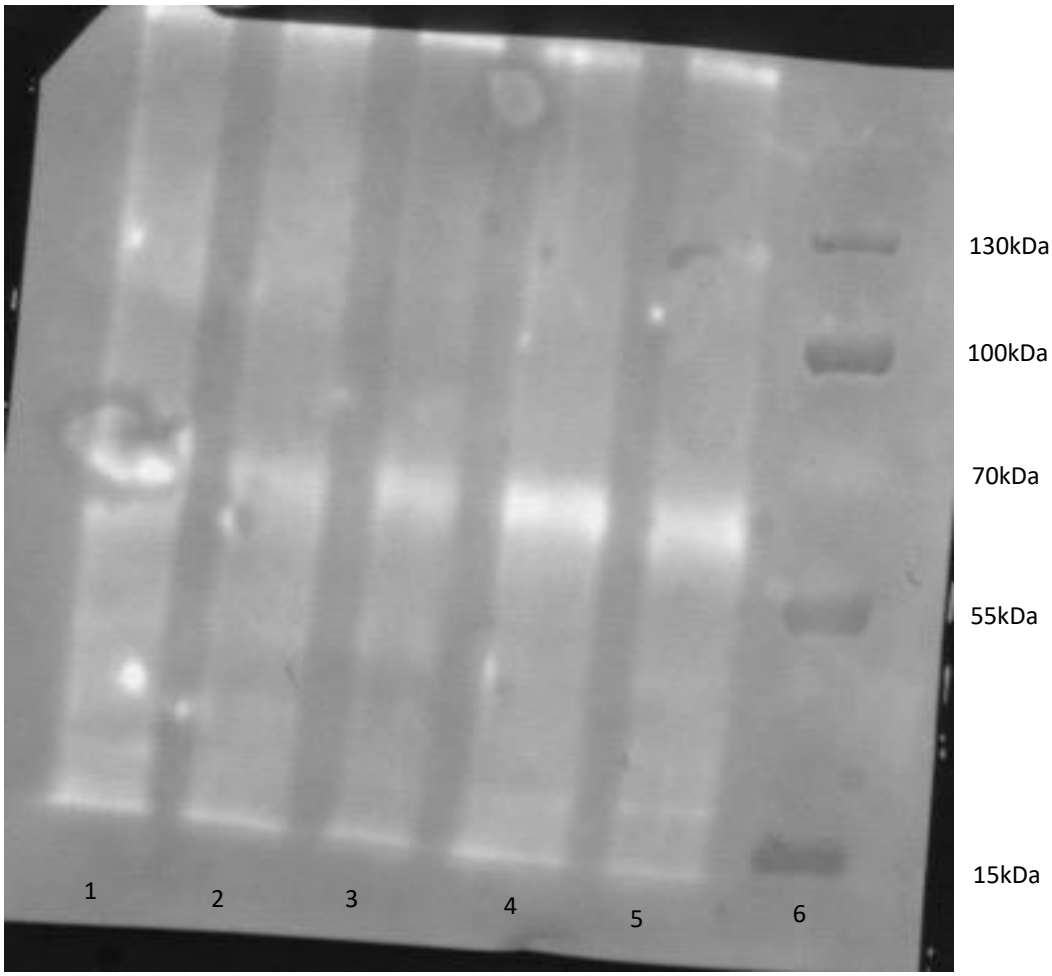


Figure 44; To explore the presence of p62 in the whole brain homogenate. Lane 1 Human brain homogenate AD (15.20). Lane 2 Human brain homogenate AD (17.07). Lane 3 Human brain homogenate control (15.30). Lane 4 Human brain homogenate control (15.31) lane 5 Human brain homogenate control (15.26). Lane 6 contained the protein molecular weight ladder.

In lanes 1-5 there is a band present just above the 55kDa region, p62 has a molecular weight of around 62kDa and therefore this is a clear indication that p62 is present within the whole brain homogenate with collagenase.

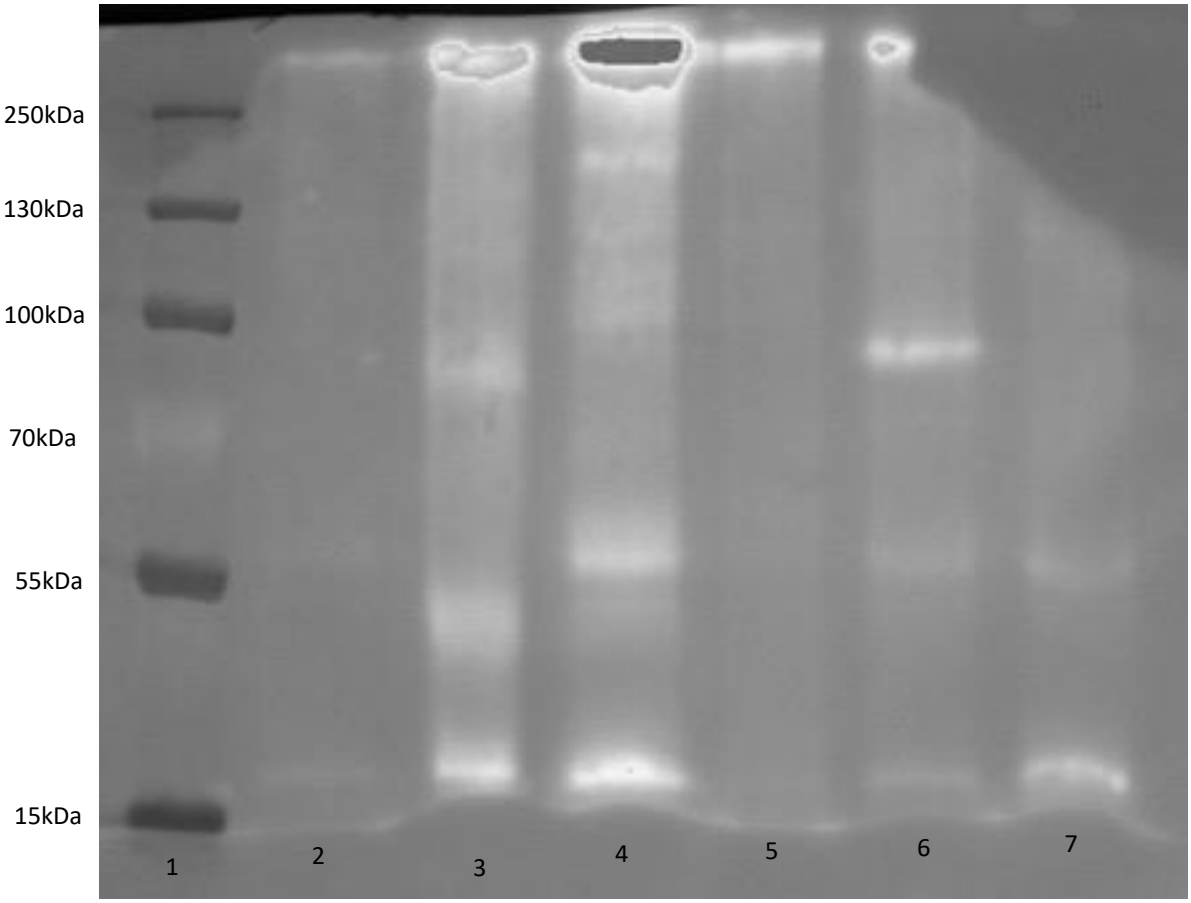


Figure 45; To investigate the presence of p62 within the sEV fractions. Lane 1 protein molecular weight ladder. Lane 2; Human brain sEV extract control (15/26). Lane 3 Human brain sEV extract control (15/31). Lane 4 Human brain sEV extract control (15/30). Lane 5 Human brain sEV extract AD (17.07). Lane 6 Human brain sEV extract AD (15.08). Lane7: Human brain sEV extract AD (15.20).

Lane 2 and lane 5 does not contain any bands therefore, no p62 is present. Lanes 3 contains 3 bands one around 15kDa, one below 55kDa and one just above 70kDa therefore, this represents non-specific binding and is not a clear indication of p62 being expressed. Lanes 4, 6 and 7 contain a band just above the 55kDa region, p62 has a molecular weight of 62kDa and therefore, the bands within these lanes show an indication of p62 being present. Lanes 4, 6 and 7 also show a band at 15kDa this is non-specific binding. Lane 6 also has a band within the 100kDa which does not represent p62.

3.5.3 Western Blots- Beta Actin

Beta actin is an antibody with a molecular weight of 42kDa, beta actin is used as a sample loading control. Sample loading controls are used in western blots to ensure the same amount of protein content is loaded into each of the wells. This chapter of results was to measure the cargo of protein in different braak staging as well as to see the presence of beta actin in small extracellular vesicle, this section was achieved by stripping and re-probing from previous experiments.

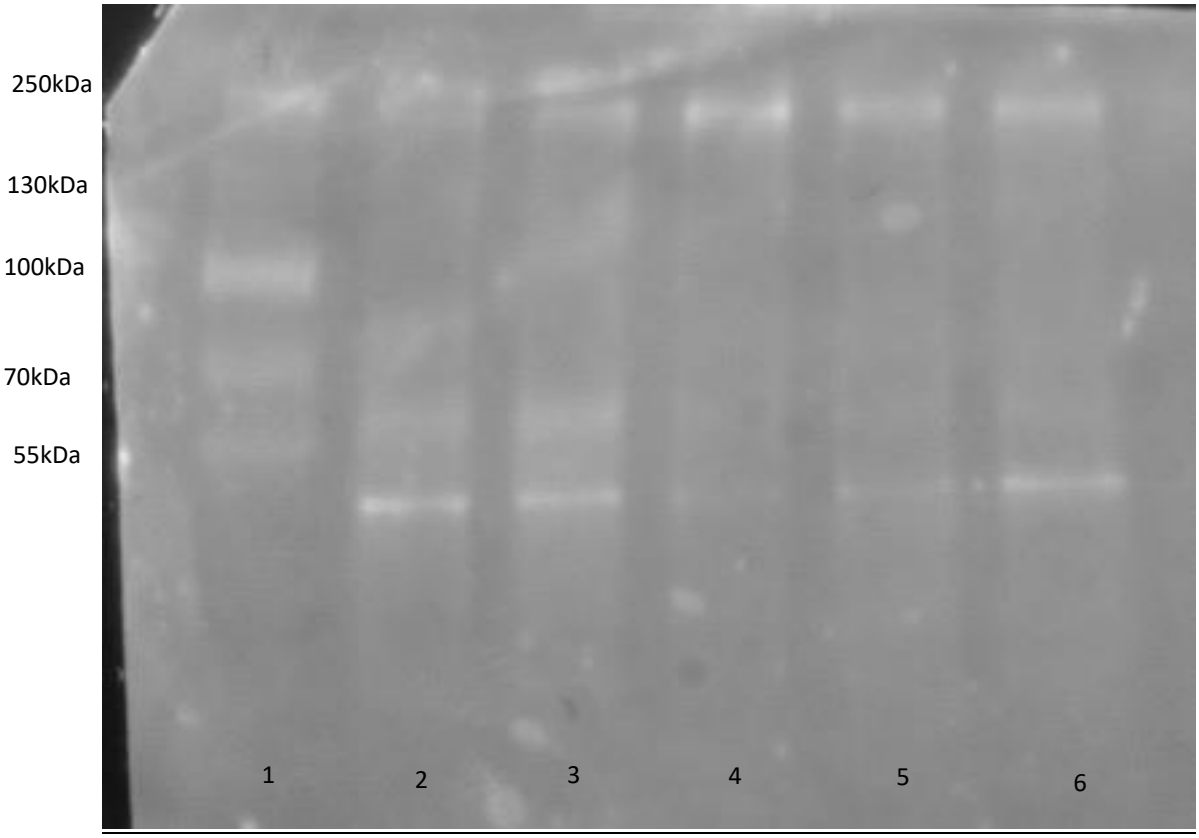


Figure 46; To explore the presence of beta actin in the whole brain homogenate with collagenase samples. Lane 1 protein weight marker. Lane 2 Human brain homogenate AD (17.07). Lane 3 Human brain homogenate control (15.30). Lane 4 Human brain homogenate control (15.31) lane 5 Human brain homogenate control (15.26). Lane 6 contained the protein molecular weight ladder.

In lanes 2, 3, 5 and 6 there were very clear bands present below the 55kDa region, beta actin has a molecular weight of 42kDa. The band is lane 4 is not as clear as the other but there is a faint band present in the correct region. The bands were within the correct region for beta-actin and is therefore a clear indication of beta actin being present within the BHC samples.

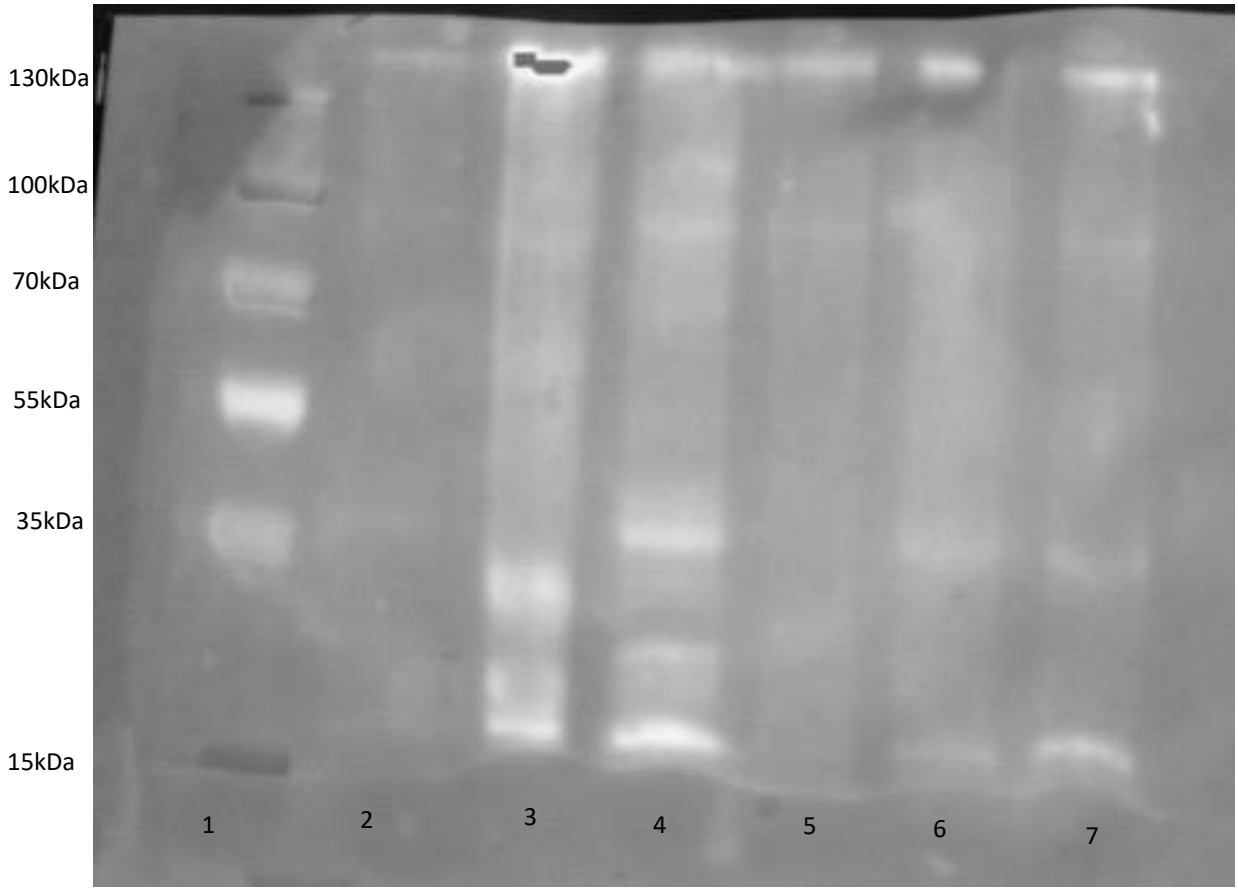


Figure 47; To investigate the presence of beta actin in the sEV fractions. Lane 1 molecular weight marker. Lane 2; Human brain sEV extract control (15/26). Lane 3 Human brain sEV extract control (15/31). Lane 4 Human brain sEV extract control (15/30). Lane 5 Human brain sEV extract AD (17.07). Lane 6 Human brain sEV extract AD (15.08). Lane7: Human brain sEV extract AD (15.20).

There were no bands present within lane 3 or lane 5 however, in lanes 4, 6 and 7 there is a faint band just above the 35kDa region. Beta actin has a molecular weight of 42kDa and therefore, the bands present within lanes, 4, 6 and 7 are a clear indication of beta actin. Furthermore, this experiment is evidence that sEVs do express the loading control beta actin. There are other bands present within the lanes at the 70-100kDa region this does not represent beta actin and is therefore, non-specific binding.

3.6 Immunohistochemistry results

3.6.1 Negative p62 stained slides

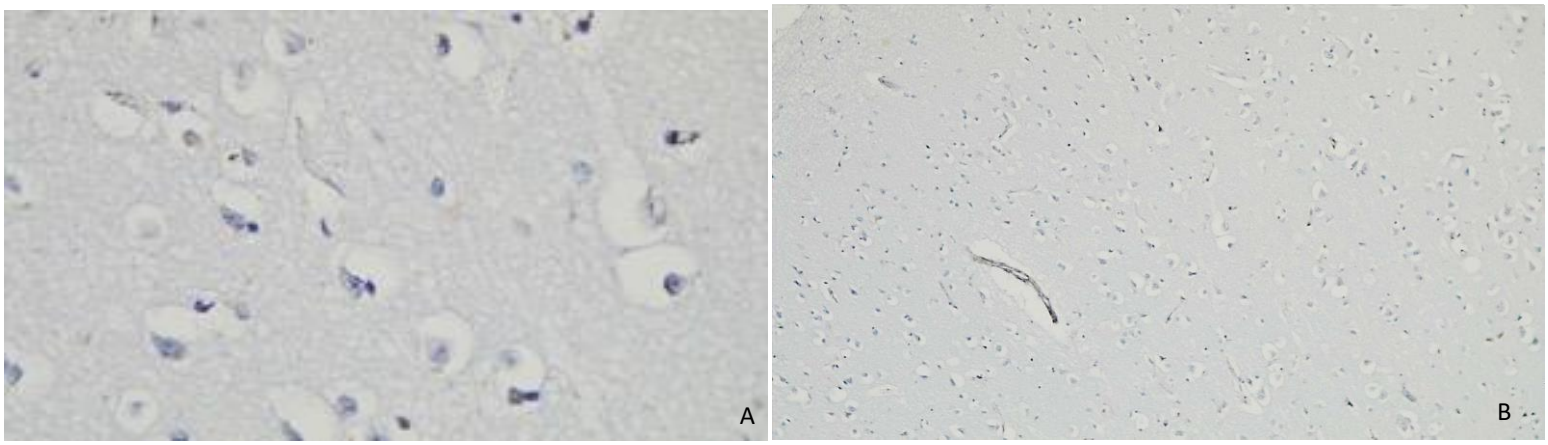


Figure 48; Negtative P62 anitbody stain of the gray matter. This brain tissue slide was from a patient in the lower braak group staging between group one & two. This slide was scored 0 for the number of cells stained and 0 for the intensity of the stain.

3.6.2 Positive P62 stained slides

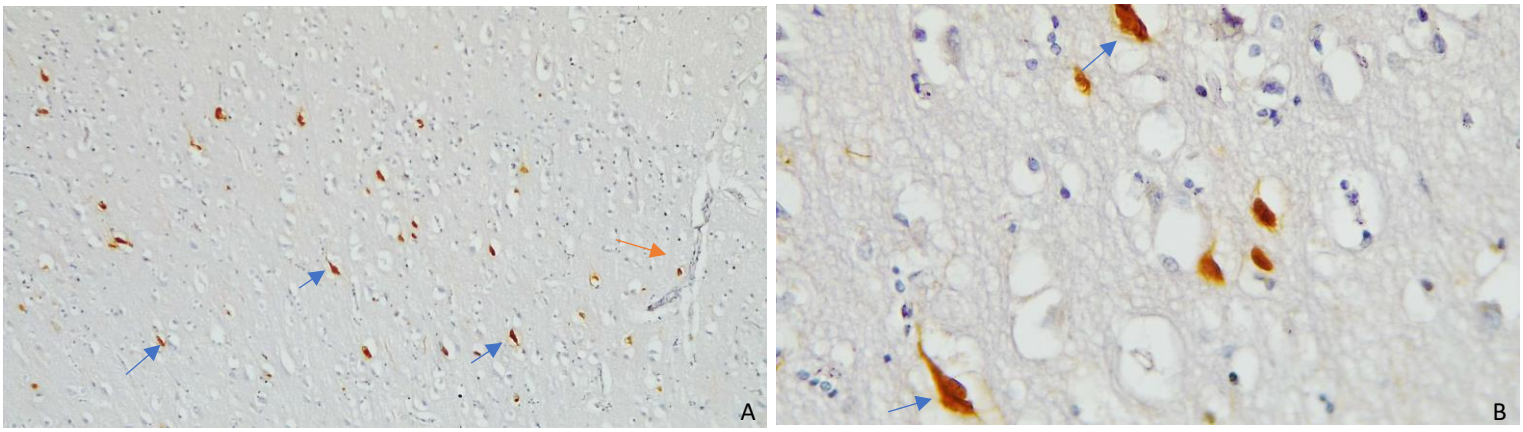


Figure 49; The intensity of the p62 stain using X100 and X400 objective. Figures A and B are positive p62 stains within the gray matter, both figures are from the same human brain tissue slide but visualised at different objective lenses. This brain tissue slide was from a patient in the higher braak group staging between group V-VI with a diagnosis of Alzheimer's disease. This slide was scored 1 for the number of cells stained and 3 for the intensity of the stain. A microvessel was captured and annoted using orange arrow and postive neurons were annoted using blue arrows.

3.6.3 Glial staining

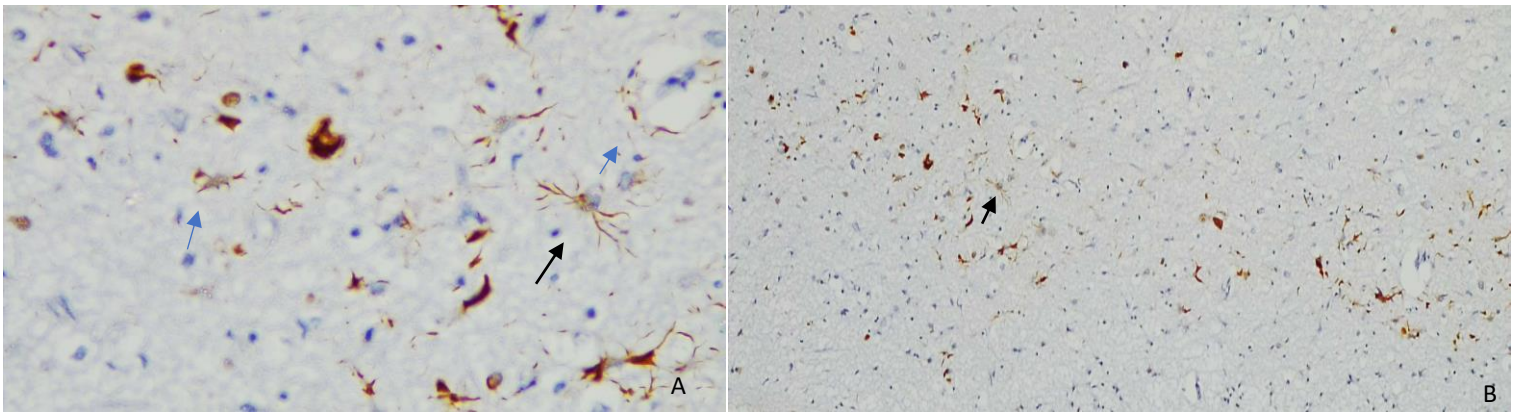


Figure 50; A positive p62 stained slide containing a reactive astrocyte at X100 objective and X400 objective. The figures are taken from the same brain tissue slide but viewed at different objectives, this brain tissue slide was from a patient in the higher braak group staging in group six with a diagnosis of Alzheimer's disease. This slide showns the presence of microgila staining - A reactive astrocyte (shown using a black arrow) as well as the presence of neurophill thread (shown using blue arrows). Image A was scored 1 for the number of cells stained and 3 for the intensity of the stain. Image B was scored 2 for the number of cells stained and 3 for the intensity of the stain.

3.6.4 AT8 stained slides

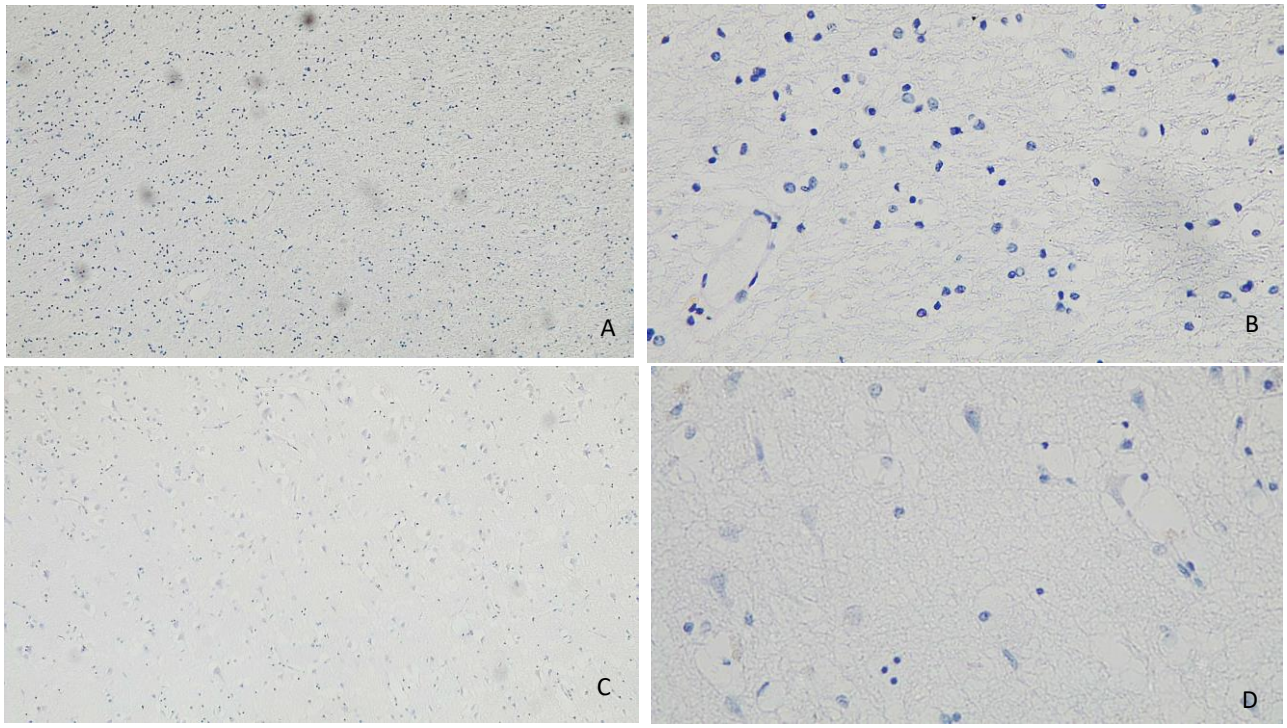


Figure 51; Negative AT8 stains in the white and gray matter of brain tissue.

Figure A shows a negative AT8 stain at X100 objective. This particular slide is the white matter area of the brain. This brain tissue slide was from a patient in the lower braak group staging in group one which is normal, no MCI or AD present. present figure B is an image taken from the same slide but visualised at 400X objective. Figure C shows a negative AT8 stain at X400 objective. This particular slide is the white matter area of the brain . This brain tissue slide was from a patient in the lower braak group staging in group one which is normal, no MCI or AD present. Figure D shows a negative AT8 stain at X400 objective. This particular slide is the gray matter area of the brain . This brain tissue slide was from a patient in the lower braak group staging in group one which is normal, no MCI or AD present.

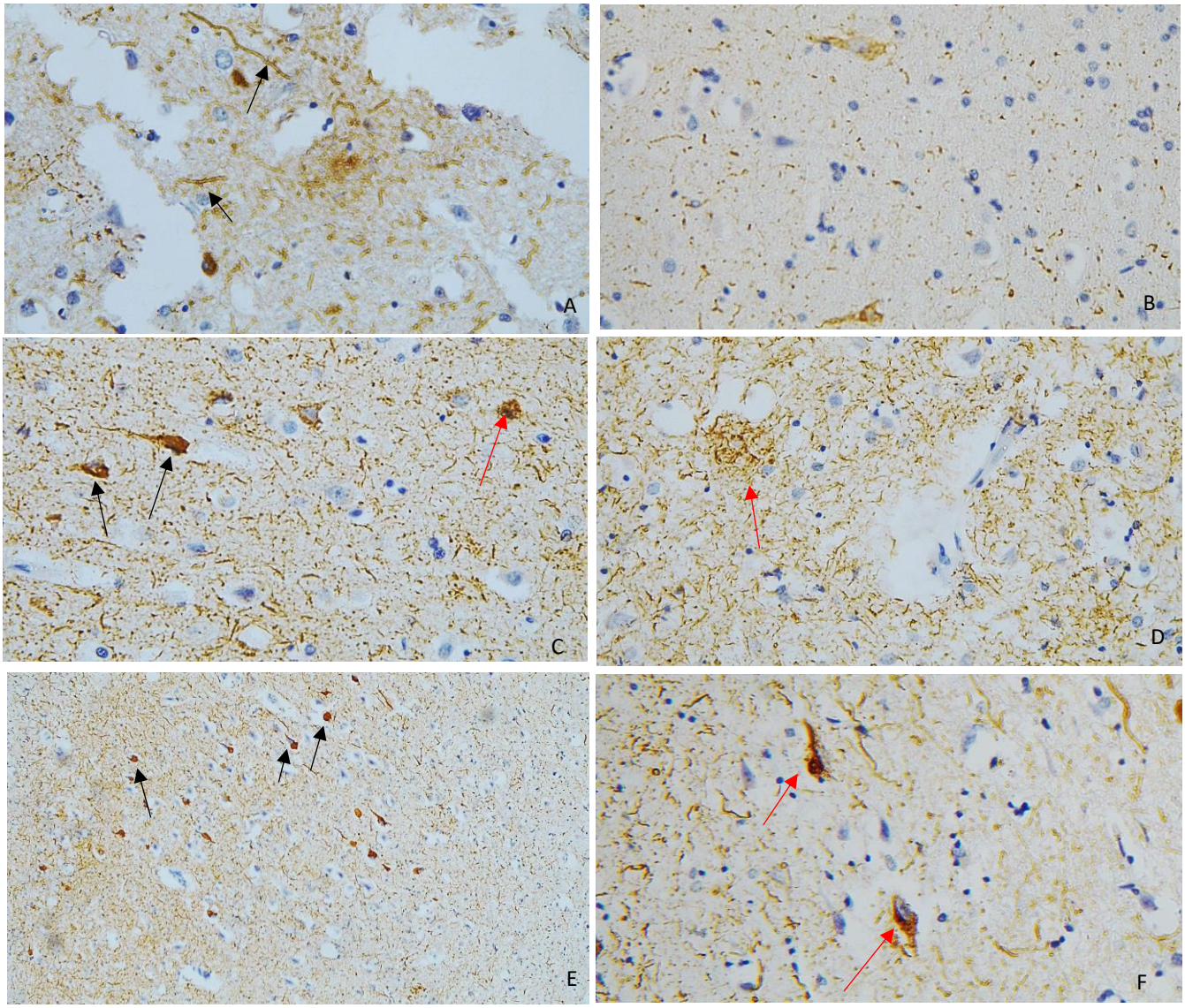


Figure 52; Positive AT8 stains across different braak groups with different intensity and different neuropathology characteristics representing AD. Images, A, B, D, F are taken from the same human brain tissue slide, with a diagnosis of AD in braak group V. Images C and E were taken from a different human brain tissue slide with a diagnosis of AD in braak group V-VI.

Figure A shows a positive AT8 stain at X400 objective. This particular slide is the gray matter area of the brain. In this slide there is neurophil thread present (representated by a black arrow) which are commonly seen in AD patients and is a neuropathology characteristic. Figure B shows a positive AT8 stain at X400 objective. This particular slide is the white matter area of the brain. The brown column inclusions are known as argyrophilic grain which is a common pathology seen in AD. Figure C shows a positive AT8 stain at X400 objective within the gray matter of the brain. This slide shows the presence of positive neurons are presentated using black arrows and a neuritic plaque shown using a red arrow. Figure D shows a positive AT8 stain at X400 objective within the gray matter of the brain. This slide shows the presence of a neuritic plaque shown using a red arrow. The scoring of this slide; cells 2 and Intensity 2. Figure E shows a positive AT8 stain at X100 objective. This particular slide is the gray matter area of the brain and is scored a 3 for the number of cells stained a 3 for the intesnity of the stain. These neurons present on this particular slide are ballooned neurons, the shape of these neurons are round - black arrows pointing at pathology. Figure F shows a positive AT8 stain at X400 objective. This slide contains lots of neurophil thread and 2 cells have been stained representing amlyoid beta (shown using red arrows).

3.6.5 P62/ Sequestsome 1 (SQSTM1) Stastical anaylsis

Autophagy is the self clearance process for protein degradation, proteins which are misfolded, damaged or old are cleared out via the autophagy system. p62 is an autophagy marker that ressembles the first initial stages of autophagy and P62 also co-localises with protein aggregates. These experiments aimed to investigate the presence, the amount and the itensity of p62 antibody stain across different Braak stages. The hypothesis was that as the Braak group increased the number of cells stained and the intensity of stain would

increase, due to an increase in abnormal protein accumulation. It would be expected that as more abnormal protein accumulation, the more autophagy markers presence to ressemble protein degradation.

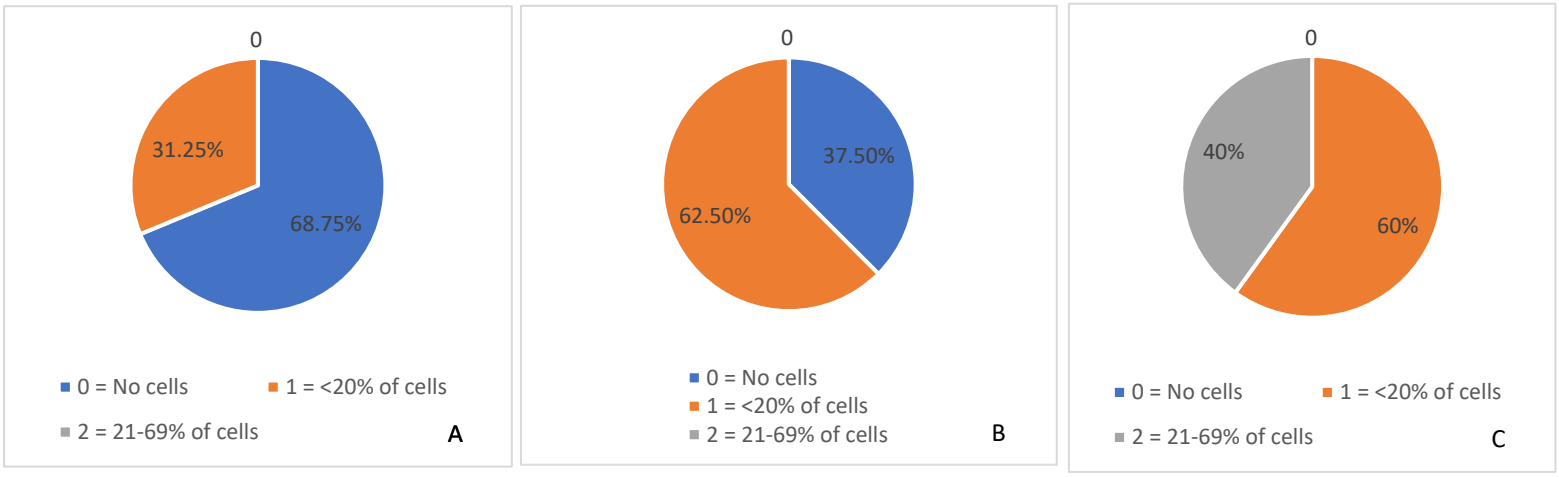
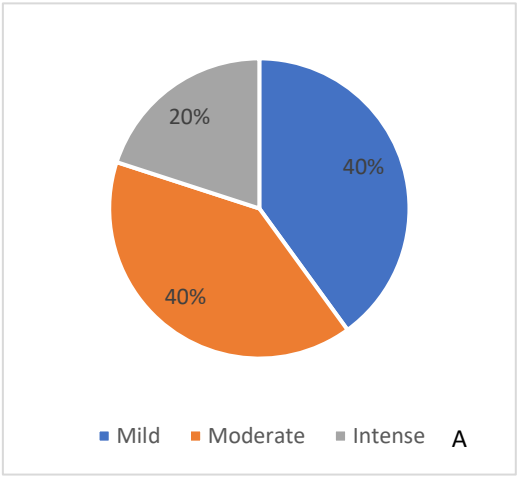
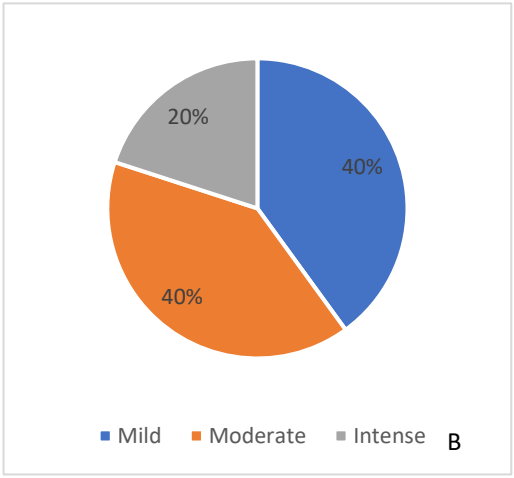


Figure 53; The total number of cells stained with p62 within the different braak groups. There were no slides scoring 3 in these studies. Image A represents braak group 0-II, image B represents braak group III-IV and image C represents braak group V-VI.

All the p62 slides were analysed and scored according to the number of cells stained across the different braak groups. FIgure A shows the % of cases that scored 0, 1 or 2 for number of cells in the lower braak group 1-2 according to the scoring guide. FIgure B shows the % of cases that scored 0, 1 or 2 for number of cells in the middle braak group 3-4 according to the scoring guide. Figure C shows the % of cases that scored 0, 1 or 2 for number of cells in the higher braak group 5-6 according to the scoring guide.





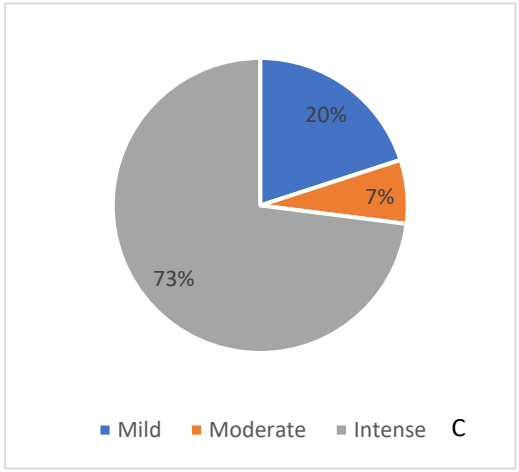


Figure 54: The intensity of the p62 stain within the different Braak groups. Image A represents braak group 0-II, image B represents braak group III-IV and image C represents braak group V-VI.

All the p62 slides were analysed and scored according to staining intensity. FIgure A shows the % of cases that scored 0 (mild), 1 (moderate) or 2(Intense) for staining intensity in the lower braak group 1-2 according to the scoring guide. Figure B shows the % of cases that scored 0 (mild), 1 (moderate) or 2(Intense) for staining intensity in the middle braak group 3-4 according to the scoring guide. Figure C shows the % of cases that 0 (mild), 1 (moderate) or 2(Intense) for staining intensity in higher braak group 5-6 according to the scoring guide.

A chi-squared test was carried out using the dichotomised data of the p62 slides available within the different braak groups. The results were dichotomised by simplifying the raw data. The slides were re-scored with either 0 and 1 therefore, slides which were previously scored a 0 or 1 become a 0 and slides which were previously scored a 2 or a 3 became a 1 this was to allow a direct assessment of 0-mild vs moderate-severe staining. The chi-squared result was; 8.65325 and the degrees of freedom were 2 which gives a P value of 0.01321. The P value is less than 0.05 which is considered to be significant therefore, this result shows that the intensity of the p62 stain becomes more intense as the braak group increases.

4.1 Discussion- declaration of issues impacting project work

In March 2020 there was a global pandemic resulting into a lockdown despite, not starting the project until May 2021 the project still experienced some drawbacks and complication due to the aftermath of Covid-19. The project was delayed, and I was not able to start any practical work until the end of July 2021, losing out on two months in the laboratory. After, getting started in the laboratory and starting experimental work there was a significant delay in shipping of materials needed to start the project. Again, in December 2021 there was a significant delay of exo-spin buffer which is an essential material needed to isolate the small extracellular vesicles from cells which took 9 weeks to arrive. This time was lost, and experiments were not able to happen due to the delays in materials. This significantly impacted the project as a total of six months in total were lost due to Covid-19. The Zetaview machine was one of the methods our group used to characterise sEVs. During my project, the machine broke and needed a software update and was down for roughly 6 weeks therefore, despite knowing how to use the NTA machine and obtaining a few results from my isolated samples; not much data from this machine was gathered. Due to this, the focus of this work was on the optimisation of processes to isolate sEVs from human brain tissue and commercially available fibroblasts, as well as Western blots characterisation of sEVs. The original imaging GBOX used in the first half of this project broke halfway through the project and was waiting for a repair. Therefore, the western blot images were not the neatest in some of the figures. Experiments could have repeated however, due to the amount of time left it was not possible and the machine was still not fixed by this point.

4.1.1 Project Discussion

Extracting sEVs from human brain tissue was possible and after adapting and after optimising the methodology created by Prof Andrew Hill, sEV were successfully isolated from human brain tissue derived from the Manchester Brain Bank. This is the first study to successfully isolate sEVs from this tissue source and will seed a number of future research projects aiming to explore the use of sEVs in early AD and FTD diagnosis, as well as a novel source of biomarkers. sEV detection was confirmed using 3 positive sEV markers; CD9, CD63

and Flotillin-1 alongside, the negative marker calnexin. This is the minimum requirement for demonstrating sEV presence as published in MISEV2018 guidelines (Théry et al, 2018).

Despite sEVs being studied in numerous disorders, they are yet to be investigated across different Braak groups and in relation to different subtypes of disease, including EOAD, LOAD and the different forms of FTD.

Despite the study demonstrating it was possible to extract sEVs from human brain tissue, sEVs were unfortunately not successfully isolated from human derived fibroblasts. Despite, undergoing multiple optimisation steps there was no positive results obtained from the fibroblasts. To date, the study of AD patient derived fibroblast sEVs has not been achieved and so further optimisation may be required, other sEV extraction methods could have been used to optimise the sEV yield. This project used ultracentrifugation with size exclusion chromatography however, other methods include antibody-based isolation, magnet-based isolation, affinity chromatograph and charge-based isolation (Witwer et al, 2013). To ensure the sample eluted is well purified and healthy a range of methods can be used in comparison to find the method which is most necessary (Witwer et al, 2013). Other sEV extraction methods are more specific and therefore, more likely to optimise the yield such as affinity-based method, studies suggest using this method isolated much purer sEV fractions (Nakai et al, 2016). sEVs have been isolated from fibroblasts in other types of patients and so this could be possible in the future, with further investment of time and resources. Given that sEVs are thought to be involved in the pathology of multiple neurodegenerative diseases including; PD, ALS, multiple sclerosis (MS) therefore, it is suggested that sEVs have great potential as a novel therapy for the treatment of neurodegenerative diseases (Xiao et al, 2021).

The western blot protocol was optimised and allowed a positive result from the human brain tissue sEV samples and therefore, indicates there may have been an issue with the sample preparation of the fibroblasts. Isolating sEVs from fibroblasts was a difficult process due to having to ensure there's enough cells present in order to secrete enough sEVs, given the sEVs were harvested from cell media rather than the cells themselves. Furthermore, there may have been issues associated with the sEV harvested from media which explains a

low sEV yield however, some studies suggest that stress inducing conditions such as; hypoxia and inflammation are used to enhance the sEV production in a number of diseases such as; breast cancer, glioblastoma, after fighting a pathogenic infection or after being exposed to an inflammatory environment (See Review Luo et al, 2021). In other studies, chemical regulators have been used to regulate and enhance the production of sEVs. The use of physical methods including ionizing radiation, ultrasound and external force have been used to increase sEV yield, it was found in one study that the sEV secretion increased significantly after radiotherapy (See Review Luo et al, 2021). However, in the process of trying to increase the production of sEV it could be causing unknown damage to the sEV as well as producing overall unreliable results.

This study was a pilot study to see if there was a possibility of isolating sEVs from both of types of samples, the aims of this project were achieved despite, not having a positive result. Given that there was only a limited amount of time and funds, further optimisation steps could not have been carried out to try and obtain a positive result from the fibroblast skin cells. However, if there were unlimited time and funds, further steps would have been carried out surrounding the sample preparation process although, due to lack of literature because of the novelty of the project there was very limited guidance. The limitations associated with the human brain tissue project despite achieving the aims, were the limited amount of tissue available from a limited number of cases, within the time period of the project.

Given there was more time available as well as brain tissue, sEVs could have been studied from an equal number of control and AD patient brains, from different brain regions (pathology and non-pathology associated) as well as from the brains of those with other types of dementia, such as FTD. The protocol developed during this project will be used to do exactly this, whilst exploring whether the cargoes of sEVs (particularly miRNAs mentioned in the introduction of this work) change between the different stages of dementia as well as the different subtypes of dementia. These variations in sEV cargoes could be used as a potential way to diagnosis disease and monitor disease progression, as well as to shed light on the underlying disease process and be used to inform therapeutic intervention. Additionally, understanding these cargoes could shed light on the pathogenic

agent contributing to the neuroanatomical spread of disease through different brain areas and help researchers interfere with this process.

4.2 Cell culture

Fibroblasts cells are commonly used in cell culture research which were first established in the 19th Century and are taken from patients using an invasive procedure known as a biopsy (Fernandes et al, 2016). Fibroblasts are important for maintaining many physiological functions within the body; synthesis of the extracellular matrix, epithelial differentiation as well as regulating inflammation and wound healing. Fibroblasts are also involved in the secretion of growth factors and involved in the scaffolding for other cell types (Tomasek et al, 2002). Fibroblast cells contain all the genetic background and can be re-programmed into stem cells. Despite, being an invasive process to collect fibroblast skin cells, it is largely used process as It is easily accessible. Fibroblast skin cells are known to be robust and are easily cultured in vitro. The use of fibroblast cell lines donated from humans via biopsy have been used in numerous studies to understand the pathogenesis of different diseases as well as being involved in disease modelling via stem cells which is important as all the genetic background from the patient is kept (Fernandes et al, 2016). Fibroblast skin cells hold many advantages and disadvantages for their use in medical research.

Fibroblast were used in this study because there are easily accessible from patients; the procedure isn't too invasive or discomforting for the patients which makes the cellular model more attractive as a biomarker in the diagnostic field (Auburger et al, 2012). The cells are robust in thawing out, sub-culturing and during transport; therefore, the characteristics will keep. Fibroblast cells keep their genetic background whilst undergoing experiment however, fibroblasts are amenable to genetic manipulation and their gene expression can be altered if necessary (Auburger et al, 2012). Fibroblast cells can be repeatedly taken from patients to monitor treatment and disease progression (Auburger et al, 2012). Despite, having some very promising advantages using fibroblasts to carry out vitro experiments they also have some limitations associated with them; the growth population of fibroblast cells are generally quite slow which was seen in this study whilst

using fibroblast cells as a vitro model. To prevent the risk of phenotypic changes; the amount of FBS used within the media was increased to 15% rather than 10% to ensure the growth rate of cells were consistent. Adherent cells are a cellular model in 2D cell culture therefore, the cells don't receive the same support and cell to cell communication they would receive within the body therefore, the results are not always accurate. In addition, cells in vitro have trophic support unlike neurons in vivo have to compete for it (Auburger et al, 2012). Contamination of cells may lead to artificial phenotypes.

The human derived fibroblast cell skins donated from AD and controlled patients were used in this study as a cellular model that has great potential of being a biomarker which is easily manipulated if needed and easily accessible sample. Dementia currently have no specific diagnostic method or any disease modifying treatments available for patient's instead; they have pharmaceuticals to treat relevant symptoms affected by the dementia therefore, this would be a massive step forward in dementia research to have an early detection method or a possibility of monitoring the disease progression. Currently, dementia is a progressive syndrome with no hope of recovering (Tsai & Boxer 2014).

4.3 Characterisation of EVs using Fibroblast skin cells.

In the first part of the study, human skin derived fibroblasts were used as a pilot study to see if there was a possibility of isolating sEVs secreted from fibroblast skin cells within the cell media. As shown in the figures within the chapter 'Western Blot Optimisation' the presence of small EVs obtained from fibroblast skin cells, were never established. The tetraspanin markers, CD9 and CD63 were expressed in the cell lysate the positive control in some of the figures but was not expressed in the sEV fraction. The problem within this study was the number of cells within the flask therefore, the sample preparation would have needed to be optimised if there was more time. In this study there was 8 million cells present within the flask after collecting the EV containing media whereas, Guerreiro et al, 2018 plated 25 million cells from each of his cell cultures and Mathilde et al, 2021 obtained 20 million cells before taking the media (containing the small EVs) to complete the EV isolation and obtaining positive results. Another group more recently (June 2022) 'Zanzhi

Jiang' isolated sEVs from fibroblast cells obtaining a positive result from CD9, CD63 and CD81 although these results do not meet the MISEV2018 guidelines the group obtained positive results from sEV samples from fibroblasts and got the Western blot working, there is very little methodology about the process of the Western blot but given the positive results shows it was a success. Despite, not obtaining positive results for the sEV isolation from my study, the importance of optimising the Western blot process was significant for other result chapters. The Western blot optimisation process took roughly six months to complete and will be discussed in the next chapter.

4.3.1 Western Blot optimisation using fibroblast cells

Several optimisation steps were encountered to troubleshoot the absence of tetraspanin markers CD9 and CD63 in the cell lysate samples as well as within the sEV fractions on the western blot. As seen in figures 14 and 15, the cell lysate (the positive control) did not show up for the probed antibodies which leads onto the first optimisation step. The first step in the optimisation process, was to lysis open the cell lysate sample as well as the sEV samples to ensure all cell contents were lysed open, Brzozowski, 2018 lysed open sEV samples using 1% NP-40 lysis buffer however, in this study the cells and sEV samples were lysed open using RIPA buffer. A 1:1 ratio of sample and RIPA buffer was agitated on a slow rock at 4°C for half an hour before use.

The preparation of the samples was the next thing to optimise and when referring to the literature of how to prepare samples prior to loading it was very mixed. Gelibter et al, 2022, prepares samples and boils at 100°C for 5 minutes, Mathilde et al, 2021 prepares samples and boils samples at 95 °C for 5 minutes and Brzozowski, 2018, prepares samples and heats samples at 70°C for 5 minutes. However, during this project the samples were heated for 70°C for 10 minutes due to study from Schulz, 2020, that states how the temperature and duration period could affect the particle count within samples. Schulz, 2020 found within their study that the particle concentration of sEVs decreased the higher the temperature and the longer the incubation period, it was found the particle concentration of sEVs decreased by tenfold when boiled at 100°C as well as resulting in chemical alterations.

The next optimisation steps are shown in figures 17-19 where the antibody dilutions along with the use of DDT added to the NUpage LDS sample buffer [x4] was investigated as it seemed the EV samples may have been getting stuck at the top of the gel. However, it seemed the addition of DDT did not solve this problem as shown in figure 17. The addition of DDT was thought to make the sample buffer a reducing agent. Methods used in Gelibter et al, 2022 and Mathilde, 2021 used a different sample buffer; Laemmli buffer which is compatible will SDS page gels used in this project unfortunately the NUPage sample buffer was not compatible in SDS pages and therefore, could have been one of the issues with not obtaining a positive result. Therefore, in figure 21 Laemmli buffer [X4] was used as sample buffer.

The antibody dilutions were optimised from reading through literature which majority suggested a 1:1000 primary antibody dilution incubated overnight at 4°C. In figure 14, a 1:1000 antibody dilution was used and there was a thick band presence in the positive control '8125 cell lysate' and a slight faint band in the '6869 cell lysate' however, in figure 15 both bands in the positive control lanes were presence, the bands were very dark and therefore, the final antibody dilution was 1:1000 for the remaining western blots throughout the project (Gelibter et al, 2022).

Brzozowski, 2018, Mathilde, 2021 and Northrop-Albrecht 2022 all highlighted with their methods which were similar in terms of duration of primary antibody; incubation period was overnight at 4°C, secondary antibody was incubated for an hour at room temperature, and 5% blocking buffer for blocking period at room temperature for an hour prior to primary antibody incubation. Therefore, due to being popular stages in majority of the literature these stages within the methods were implemented into my project and used for my western blots.

Some of the western blot stages were not optimised, the gel was run at 100V for the first half an hour allowing proteins to migrate into the resolving gel. Once the proteins migrated into the resolving gel, the voltage was turned up to 140V for an hour in a tray packed with ice at room temperature with an ice pack within the western blot tank. The transferring of the SDS page onto the nitrocellulose membrane was completed on 100V for an hour at 4°C

or 0.25V overnight at 4°C. After the primary and secondary antibody incubation the membrane was washed 3 x 10 minutes with PBST before proceeding onto the next step. The blocking buffer used in this study consisted of 5% BSA in PBST (PBS + tween 0.1%). The membrane was imaged using Super signal west Atto ultimate sensitivity chemiluminescent substrate at 1:1 ratio.

Further optimisation steps would have included increasing the number of cells present within the cell culture media to increase the rate of sEVs secretion within the flask to increase the sEV yield. An increased sEV yield would allow more protein to be loaded within the wells of SDS-page increasing the chances of a positive band. The optimisation steps already completed indicate the problem was with the sample preparation rather than the western blot process. The presence of bands within positive control samples indicate the problem was due to the lack sEVs within the sEVs sample.

4.4 Human Brain sEV characterisation

The aim of chapter was to investigate whether sEVs could be extracted from frozen human brain sections. The protocol was created by our collaborator Prof Andrew Hill and optimised by 3 members of Dr Gemma Laces' Group; Toby Aarons, Joesph Morgan and Shannon Youd. The methods used to characterise the presence of sEV were; immunoblotting and nano-particle tracking analysis using the MISEV2018 guidelines.

In this study the experimental methods used to confirm the presence of sEVs from frozen human brain sections were western blots and fluorescent nano particle tracking analysis. The western blot was optimised in the previous chapter and all the methodology was the same throughout the western blots in this chapter. To complete this study 3 AD samples were used (15.08, 15.21 & 15.20) and 3 controlled samples were used (15.30, 15.31 and 15.01). The first initial experiments were completed dependent on the amount of tissue available however, in the end the experiments were completed in biological triplicates.

sEV membranes are enriched with tetraspanins markers (CD9, CD63, CD81) as well as various other proteins like Alix and TSG101; markers identified in the biogenesis of EVs or

membrane and fusion markers Flotillin and GTpase (Khushman et al, 2017 & Tucher et al, 2018). The most common antibodies used are the tetraspanin markers; CD9 and CD63. To meet the MISEV guidelines; to characterise small EVs; 2 tetraspanins antibodies were used, CD9 and CD63 and the membrane bound protein Flotillin-1 was used to confirm the presence of sEVs using western blots.

Human brain tissue 15.20 and 15.01 only obtained a positive result for CD63 and calnexin, this membrane was cut and probed for a positive and a negative marker. However, the other human brain tissue samples (15.21, 15.08, 15.30 & 15.31) were probed for CD9, CD63, Flotillin-1 and Calnexin.

Calnexin is an endoplasmic reticulum protein, which was detected in whole brain tissue but were excluded from the sEVs samples as shown in figures 16, 21, 22 the band calnexin can be seen between 70-90kDa on a western blot. Calnexin would not be expected to be detected to in sEVs and therefore, in this experiment used as a negative marker of sEVs (Tucher et al, 2018). The absence of calnexin within the sEV fractions also tests the methodology purity. MISEV guidelines states there needs to be a negative result for a negative marker (Calnexin / GM130) in Western blots in order to complete SEV characterisation and evidence the sample was not contaminated by other fractions that would give false positives via the tetraspanin markers (Théry et al, 2018).

CD63 was a tetraspanin antibody used to confirm the presence of sEVs which appears as a smear around 30-60kDa on a western blot; as shown in figures, 21, 24, 27 & 28
Figure 27 and 28 were the same membrane, but the positive control was cut to receive a better signal and overexpose the image to get a thicker and more visual band. CD9 was another tetraspanin antibody used to confirm the presence of sEVs, which appears as a single band around 25kDa in a western blot as shown in figures 23, 29 and 30. Figures 29 and 30 were the same membrane but without the positive control, this was to overexpose the membrane to get a better signal from the sEV lanes. Flotillin-1 was another antibody used to confirm the presence of sEVs. Flotillin-1 is shown in figures 22 and 31 to confirm the presence of sEVs isolated from the human brain tissue samples.

The western blot experiments within this chapter met the MISEV2018 guidelines; the western blots confirmed the presence of 2 tetraspanins markers CD63, CD9, as well as the presence of flotillin-1 in the sEV sample lanes. The negative marker used was calnexin which did not show up in the sEV lanes but did show up in positive controls which was expected and can therefore say that sEVs were successfully isolated from human brain tissue.

To characterise the sEVs further, the samples were analysed using NTA analysis. In the first instance, the scatter mode on the zetaview shows how many particles are present within a sample. Once the scatter mode has confirmed there are particles present within the samples, the samples can be characterised using fNTA. Prior to tagging samples with the antibodies to confirm for sEVs a cell masked orange dye is used to confirm the presence of the biological particles within the samples and their sizes. Once it has been confirmed that there are biological particles within the samples, the samples can be used for characterisation purposes. The cell mask orange dye works by eliminating any non-biological particles within the samples by staining the membranes of the biological particles.

The NTA was completed 3 times using 6 samples, 3 AD and 3 control samples. The first experiments were completed using samples 15.08 (AD sample) and 15.30 sample (control) the scatter shows that there are more particles within the control sample compared to the AD sample. The AD sample had 1.2E+11 particles whereas the control sample had more particles with 1.37E+11 particles present. The number of biological particles were significantly higher in AD sample 15.08 compared to control sample 15.30, the vast majority of particles from both samples were in the sEV range. There were no CD markers detected with these samples and this could have been due to the duration period of the incubation, manufacturing recommendations state a minimum of an hour therefore, on the next set of experiments the antibody and sample incubation period was increased to a minimum of 2 hours to try and detect more particles tagged with CD9, CD63 and CD81 despite, other studies incubating for less time (Thane, Davis & Hoffman, 2019) in this study the incubation was increased and with further optimisation steps it eventually worked.

The second experiments were completed using brain tissue samples 15.20 and 15.01, the scatter results show that the number of biological particles were significantly higher in the

control sample compared to the AD sample. The AD sample had 2.7E+10 particles present whereas the control sample had 8.73E+10 particles present. The controlled sample obtained a positive result for CD9 but not for any of the other CD markers. The AD sample did not obtain any positive results for the CD markers but did for the CMO. The CMO shown that there were biological particles present within the sample within the sEV range despite, not showing a positive result for the CD markers. Due to the lack of results for the CD markers, the dilutions were changed to try and optimise the number of particles detected. The averaged sized biological particles in AD sample 15.20 were 277.4nm and 282.7nm within the controlled sample 15.01. The means of the biological particles are not within the sEV size range however, this is due to lack of particles present within the sample. The average size CD9 particles were 444.8nm in sample 15.20 and 202.4nm in the 15.01 controlled sample, the average size of the particles is a lot higher in the AD sample compared to the controlled sample, this is due to lack of particles within the sample.

The final experiment was completed using brain tissue samples 15.31(AD) and 15.21 (control). The scatter results shown that there were more particles within the controlled sample 15.31 compared to the AD sample 15.21. The number of particles present within the AD are 3.76E+10 whereas the controlled sample contains 4.3E+10 particles. The number of biological particles present within the AD sample was considerably more than the control sample. The mean size of the particles in 15.21 is 180.6nm and the mean size of the particles within the 15.31 is 193.7nm despite, the average sized particles being smaller within the AD sample both of the averages are within the sEV size range. The mean size of the particles tagged with CD9 were 118.6nm in the controlled sample and 122.8nm in the AD sample which differs from the mean sizes in the biological particles. The mean sized particles in the CD9 experiments are within the sEV size range and shows that sEVs were successfully isolated and were present within these samples.

By visually looking at the scatter graphs, it shows that there are more particles present within the control samples than the AD samples, to check for any variations within the disease groups a T-test was carried out. The t-test had a P value of 0.53 and therefore, means there is no variation between the disease groups a value above 0.05 means insignificant and therefore, cannot say that all control samples will contain more particles

than AD samples. The average size of the biological particles visually shown that the biological particles in the AD samples are smaller compared to the biological particles within the control samples however, a t-test shown a P value of 0.70 which is insignificant meaning there is no variation between the disease groups. The average sized particles tagged with CD9 antibody shows that the particles are bigger in the control samples compared to those in the AD samples however, the t-test had a P value of 0.38 which means it is insignificant and there is no variation between the disease groups. Due to all the P values being insignificant this shows that no further tests need to be done to compare the variation between the disease groups. However, the data shown is not due to physiology itself due to optimisation the starting volumes may be different and therefore, could alter the overall P values. Therefore, this is not enough to determine if there is variation between the disease groups and experiments would need to be repeated and further research would be needed in order for this statement to be made. However, this data does show that fNTA can be used as a characterisation method for sEVs.

Despite, not all CD markers working for the samples and not all the samples containing markers there was some results which contained CD markers and with further optimisation steps this methodology could help further sEV research to potentially become a biomarker by investigating the total concentration of particles and the size distribution between of sEVs from different samples within disease groups to see whether there is any variation which could be used as an early detection method (Frühbeis et al, 2015 & Arraud et al, 2014).

4.5 Pathogenic protein marker AT8;

One of the pathogenic hallmark proteins associated with AD is hyperphosphorylated tau. The abnormal build-up of tau fibres within the neurons causes neurofibrillary tangles (NFTs) which is known to cause neuronal death due to the lack of physical and physiological function leading onto the critical symptoms of AD. AD is a progressive disease which gets significantly worse over time (Miao et al 2019). The abnormal accumulation of tau has a positive correlation with disease progression, it is known that as AD progressive the more tau in present within the brain which eventually spreads to all lobes in the brain which is

characterised using the braak staging method. This is a method which is used to measure the degree of pathology in AD and the localisation of the tau. The braak staging is a diagnostic in AD at autopsy (Therriault et al, 2022).

This chapter focuses on the use of sEVs as a potential biomarker using western blots, in this study six different samples were investigated for AT8 (hyperphosphorylated tau antibody), three of the samples were from the lower Braak group (1-2) considered to be mild or an aged brain and three samples were from the higher Braak group (5-6) considered to be severe AD cases in hope to see a difference between tau levels within the lower and higher Braak group staging.

The western blots were set out that the first three lanes were samples from the lower Braak group and the last three lanes were samples from a higher Braak group, the expected outcome of these results with the literature stating the positive correlation between the amount of tau and disease progression was to see a thicker band in the higher braak groups compared to the lower Braak groups. AT8 (Phospho-Tau Ser202, Thr205, thermofisher) was used as the primary antibody to detect hyperphosphorylated tau in the BHCs as well as small EVs. An antibody dilution of 1:500 was used with 2.5% BSA and PBST.

Despite, seeing positive AT8 results in the literature in other cellular models, the results obtained within the study were not the best results. The antibody needed optimising; the protein load would have needed to be increased in order to see a definitive band as well as increasing the BSA to 5% in the primary rather than only using 2.5% BSA (Stojakovic et al, 2021 & Aulston et al, 2019).

To confirm the presence of AT8, there should be a band presence around the 50-60kDa mark, in figures 40 and 41 there are no bands present along the membrane only the top of the wells, given that AT8 is found in aggregated tau the bands at the top of the membrane could identify as aggregates which prevented movement though the gel. Phosphorylation impacts the ability of tau to move through the gel- using lambda protein phosphatase treatment to dephosphorylate tau would a have allowed visualisation of tau using a different marker such as those to 3R and 4R tau.

The imaging machine used was different to the ones used prior and therefore, the images received from the visualiser were not the best. In addition, the antibody may need optimising further before using again. The western blot in figure 40 is a very messy image and therefore, there is no distinctive bands presence. There seems to be a linear line present where you would expect to see the bands however, using this image it cannot be sure that this band relates to AT8. There was a human error made when loading the gel, 2 X 15.08 samples were loaded instead of only one. Female and male mice have been used to investigate AT8 and western blot shows a positive band for AT8 in mice therefore, it is expected for the AT8 antibody to be compatible with human brain as a cellular model (Stojakovic et al, 2021). However, due to there being a number of varies tauopathies this could indicate, this type of tau was not detectable within these brain sections. Despite, not seeing a positive AT8 band for the BHCs when probing the sEVs for A8, there was a clear definitive band in some of the samples as shown in figure 43, an increase of protein load would have made the bands more distinctive. Aulston 2019 investigated the presence of AT8 in small EVs isolated from mouse brains within this study there was a clear band in the PS1 (presenilin-1 mutation) and NDC (non-disease control). There were more bands present in the AD mouse samples compared to the control group however, there was a positive presence of AT8 obtained from sEVs, this symbolises that sEVs would cause hyperphosphorylated tau within their protein cargo linking back to the hypothesis that sEVs could potentially acting as a prion and spreading pathogenic tau around the brain in-between neurons, causing healthy neurons to become pathogenic (Schneider et al, 2018).

4.5.1 Initial autophagy marker; p62

Autophagy is the self-clearance system of dead organelles, waste and misfolded proteins. Autophagy is a significant system when it comes to neurodegenerative diseases, many researchers are trying to understand what happens to the autophagy systems during neurodegenerative diseases. It is thought that if the autophagy system was carrying out its role effectively diseases like AD and FTD would not exist however, it is also thought that the system isn't able to work effectively due to being so overwhelmed by the abnormal protein accumulation (Glick, Barth & Macleod, 2010). The literature shares the important of

autophagy in the investigation of beclin-1 levels, the decrease in beclin-1 levels show an increased risk of neurodegenerative diseases thus, could be used as a potential therapeutic. However, this study focuses on the use of finding a biomarker to diagnosis neurodegenerative diseases through the presence levels of autophagy markers; this project focuses on the p62 marker.

This study uses the initial autophagy marker p62, to try and understand the process and relationship between the autophagy markers and neurodegenerative diseases and if the levels of autophagy show significance. The first experiment using the immunoblotting technique; western blot to confirm the presence of p62 within the whole brain homogenate with collagenase (BHC) sample and the sEVs. The samples from both the BHC and sEVs were isolated from three patients without a diagnosis of AD considered to only have age-related changes and three patients with a diagnosis of AD considered to have a diagnosis of severe AD from the braak stage analysis.

The hypothesis of this experiment was a contradicting one, as literature shown as AD progressive there's an increase of tau presence therefore, it was thought that as the levels of tau increase so would p62 as this is an initial autophagy marker. The more tau presence the more p62 present, to start of the autophagy process of other markers; LC3 to clear out the unwanted abnormal protein. However, it was also a thought that the levels of p62 decrease due to other autophagy markers being present to effectively get rid of the debris. The results from the western blots were not able to conclude this hypothesis due to an unequal amount of protein load proved using beta actin which is shown in figure 46 and 47. However, the levels of p62 in different stages of AD will be discussed further in the next chapter; 'Immunohistochemistry'.

Despite, not being able to measure the levels of p62 in the different Braak groups using western blots, the results from the immunoblotting experiments still show significant results; in figure 44 the western blot show the presence of p62 within the BHCs which would be expected as p62 is also involved the maintenance of the cellular homeostasis as well as involved in regulating the degradation of waste proteins (Lui et al, 2016 & Caccamo et al, 2017). The presence of p62 in human brain tissue is also shown in the

'Immunohistochemistry' chapter. The result saw in figure 45 show non-specific binding as the bands within the sEV lanes have different molecular weights. However, in lanes 4, 6 and 7 there are bands in the expected region which could indicate the presence of p62 within the sEV samples. The autophagy marker p62 does not seemed to have been investigated in extracellular vesicles therefore, further experiments would be needed to evidently say if the sEV cargo shows the autophagy marker p62. Furthermore, other autophagy markers such as; LAMP2A and beclin-1 could be investigated within the sEV fractions to differentiate between the different types of autophagy within different disease groups.

4.6.2 Beta Actin- loading control

Beta actin was used as a positive sample loading control within the AT8 and p62 experiments, the results from the western blot within the figures 46 and 47 show there was some inconsistency with the amount of protein loaded into wells due to band thickness changing between the lanes as shown In figure 46.

In order, to quantify the levels of antibody all the bands within the beta actin experiment must all be equal to show, the amount of protein loaded was the same and changes in bands are due to the levels changing within the samples. In order to work out the protein concentration and how much to load onto the SDS page, prior to the western blot a BCA was carried out however, the amount of sample used was minimal, in these experiments a 1:80 dilution was used. Therefore, due to such a minimal amount the protein being used the BCA results were not the most reliable and this is shown via the western blots experiments in figure 46. However, it could have also been the buffers that were used in the sEV extraction process interfering with the BCA therefore, the outcome of results may have been inaccurate causing unequal loading of protein within the western blot meaning the levels of protein were not comparable between lanes.

To investigate whether sEVs express beta actin, the sEV fractions membrane was stripped and re-probed using beta actin antibody and visualised, the results obtained from this experiments were not the cleanest of images and is therefore, hard to say whether or not sEVs express beta actin and therefore, further research would be needed to be carried out.

4.6 Immunohistochemistry; AT8, P62

The immunohistochemistry experiments were to check the with the literature hypothesis but also to be able to recognise basic neuropathology. When looking through the slides for positive and negative AT8 (tau) slides, the basics of neuropathology were learnt and visualised.

4.6.1 AT8 neuropathology

Whilst looking through the slides, the neuropathology characteristics have been annotated; positive and negative neurons, ballooned neurons (BN), white and gray matter as well as some AD pathologies; neurofibrillary tangles (NFTs), argyrophilic grains, neurophil threads and neuritic plaques. There are three types of tau lesions; neurofibrillary tangles, neurophil threads and neuritic plaques which are all caused by the abnormal protein build-up tau (Lam et al, 2022). Neurophil threads pathology are shown in figure 52 image A with a black arrow. Argyrophilic grains are also another common neuropathology seen in the brain of patients with AD which is shown in figure 52, B which can only been seen during post-mortem. Argyrophilic grains disease in highly frequent sporadic tauopathy which is under-recongnised and is most commonly seen in patients with AD or other types of neurodegnerative diseases (Rodriguez & Grinberg, 2015). Studies have revealed that hyperphosphorylated-tau protein is responsible for AGD and is frequently saw overlapping with AD pathology. Due to the major overlap with AGD and AD pathology it makes it hard to thoroughly investigate the role of AGD in cognitive decline (Rodriguez & Grinberg, 2015). In figure 52 image E, the positive neurons are also known as ballooned neurons. Ballooned neurons are associated with different types of neurodegenerative diseases and demenita. Ballooned neurons are more commonly assiocated with picks disease also known as FTD, corticobasal degeneration and argyrophilic grain disease (AGD) as well as being seen in AD but the freqency of BNs in AD has not been address as of yet (Fujino et al, 2004). Initially, the aim of this project was to match IHC stainned sections with western blotting data from the same case however, there wasn't frozen tissue availble to allow this. In the future pathogenic protein markers could be invesitgated via western blot from patient sEVs via to help differentiate between diffrerent types of dementia as well as investigating the sEV

cargo as the disease progresses. If sEV cargo levels do change as disease progression this could be a method of early detection to diagnosis dementia early.

4.6.2 P62 staining and stastical tests;

In AD p62 attempts to degrade tau by binding onto the NFTS (Caccamo et al, 2017) therefore the hypothesis is that, if the number of NFTS increases due to disease progression there must be an increase in p62, as there is more tau that needs degrading. As discussed in the 'Initial autophagy marker; p62' chapter from the discussion section, the hypothesis of whether the levels of p62 increases as the disease progresses or not is still an unknown in this study due to obtaining unreliable results from the western blots. However, using immunohistochemistry this hypothesis for these particular cases were able to be identified. In figure 48 the slides show a negative p62 antibody stain, the human brain section used for this task was taken from a patient in the lower braak group considered to only show age-related changes. However, in figure 49 show a positive p62 antibody stain, the human brain tissue used on this slide was taken from a patient in the higher braak group 5-6 and considered to have severe AD. The stain in figures 49 is considered to be an intense stain. The intensity of the stain is suggested to be either, mild moderate or intense. The more intense the stain is, the more of the antibody is present.

To conclude the hypothesis, all the slides available with p62 stain across the different braak groups were scored according to the scoring guide the raw data was dichotomised and a chi-squared test was carried out to test the hypothesis, if the intensity of the p62 becomes more intense as the braak group increases. The chi-squared result was; 8.65325 and the degrees of freedom were 2 which gives a P value of 0.01321. The P value is less than 0.05 which is classed as being significant therefore, this means the null hypothesis can be rejected and the experimental hypothesis accepted. Despite obtaining a P value some of the expected data inputted into the chi-square test was less than 5 and is therefore, deemed as being unreliable. In order to make this statement and rely on the results from the chi-squared test further research would be needed.

4.7 Limitations of study

The first limitation of this study, was the use of 2D cell culture despite, 2D cell culture being the most popular cellular model to use in research due to the well established and inexpensive costs compared to other cellular models. The use of 2D cell culture does not give an accurate representation of how cells grow, interact or how they react to disease due to the physiological conditions being different in a 2D environment compared to the human body. Due to the study being novel, there was very little information regarding obtaining sEVs from fibroblasts which took a lot of time to optimise. The time was mostly spent trying to optimise the western blot process rather than anything else. Another limiation of this study was the use of human brain tissue despite, obtaining very exciting results the human brain tissue can only be donated after post-moterm there is no other way of obtaining human brain sample before death, due to being a very invasive and life changing process and would not be an ideal method of becoming an early detection biomarker for diganosising AD but the results obtained from the human brain tissue may correlate to the end outcome. Not only is it an invasive method, the human brain tissue is considered to be a precious sample therefore, there was only a limited amount of tissue avaliable for this project. However, investigating this tissue which is at the focus of neuropathological change, could allow the identification of novel candidates for further investigation in peripheral tissues.

4.8 Future work

One of the difficulties with diagnosising and treating AD is that, there is thought to have 20 different tauiopathies however, there is currently no method to disguish between the different types of tauiopathies and which tauiopathies is causing the disease to be able to understand the clinical and pathology features of them (until post-mortem). Therefore, one of the future experiments which would be interesting to carry out is, to use the brain tissue previously used, using western blots to probe for different types of tau within the controlled human brain samples and the diseased human brain tissue, to investgate the tau present and the levels of tau. Given that, sEVs are thought to transmit the tau protein from diseased neurons to healthy ones it would be interesting to investigate the types of tau present in the

sEV samples replicate those detected in whole brain homoegnate or slides stained by IHC. Furthermore, another research question is to understand how the sEVs cross the blood brain barrier. Another interesting experiment would be to isolate sEVs from human blood samples, to invesitgate the use as a potential biomarker - comparing levels of sEVs in controlled patients VS AD patients, but to also investigate if variations identified between neurodegenerative disease groups using brain tissue, are also present within the blood samples, which are readily accessible.

Autophagy is the system to remove dead proteins and waste material from the body, and it would be interesting to induce autophagy to cell culture models (using drugs such as rapamycin) to see the potential changes in sEV levels and cargoes, as well as looking for a reduction in tau levels after inducing autophagy within the cell lysate and sEV samples.

5.0 Conclusion

This study was a pilot study aimed to investigate whether sEVs could be extracted from 2 different AD associated sources; human brain tissue and commercially available AD patient derived fibroblast cells. The aims of this study were achieved, despite not being able to isolate sEVs from fibroblast skin cells as the methodology was trialled and optimised to help further research studies. The results obtained from the human brain tissue positively showed that sEVs can be extracted from human brain tissue and the methodology was adapted accordingly from Professor Andrew Hill group. The sEV fractions isolated from the human brain tissue shown the presence of autophagy marker p62, despite not having a positive result from the AT8 experiments which may have been due to how samples were prepared. Studying sEV cargoes in the first instance in human brain tissue to identify candidates that may help differentiate between different types of dementia, then following this up using peripheral tissue such as blood, could identify novel biomarkers for use in early and precise disease diagnosis. The early detection of disease in a way that also sheds light on the underlying disease driver, could reshape dementia patient care and therapeutic intervention.

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