Clusterin modulates amyloid-β peptide levels without affecting their production

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Preface and contribution of authors

The work performed in this thesis builds on the cell-based biochemical protocols for SDS-PAGE, Western blot, and immunoprecipitation established by a previous Master's student, Travis Rilea. Travis also provided the HEK-Clu stable cell line used during this thesis. A previous PhD student from the Multhaup lab, Dr. Filip Liebsch, created the SH-SY5Y-Mock, -APP, and -BACE1 stable cell-lines used in sections 3.2 and 3.3. Dr. Mark Wilson of University of Wollongong provided the G7 hybridoma antibody producing cells used to generate the anti-clusterin antibody for Western blot detection of clusterin.

I designed and performed all other experiment in this thesis under the supervision of Dr. Multhaup, with the input and suggestions of all Multhaup lab members and Dr. Mark Hancock from McGill University.

1. Abstract

Alzheimer disease (AD) is the world's most common form of dementia (43.8 million affected as of 2016) and, due to rapidly aging populations, the incidence and socioeconomic burden of this disease is expected to dramatically increase by 2050 (projection is 100 million affected). A critical event in the pathogenesis of AD is the accumulation of amyloid-beta (Aβ) peptides as toxic Aβ oligomers in the brain which ultimately lead to neuronal damage and synaptic loss. Increased Aβ peptide accumulation can occur through two possible routes: (1) the over production of Aβ from the enzymatic cleavage of amyloid precursor protein (APP) or (2) a decrease in the degradation and clearance of Aβ peptides. Clusterin is an extracellular chaperone linked to sporadic AD through genome-wide association studies, but its role in the pathogenesis of AD remains unresolved. Therefore, my thesis sought to examine the effects of clusterin on Aβ peptide production versus degradation. The clusterin overexpression experiments resulted in increased Aβ peptide levels, an effect that was independent of Aβ production since clusterin failed to modulate the beta- or gamma-secretase enzymes that sequentially cleave APP. Thus, the data indicate that clusterin likely modulates the degradation and clearance of Aβ peptides. Since clusterin is up-regulated in AD, future studies are needed to determine the precise mechanism by which clusterin can increase Aβ peptide levels. If the negative impact of clusterin on amyloid degradation and clearance can be circumvented, this provides a new therapeutic strategy to combat the pathogenesis of AD.

1. Resumé

La maladie d'Alzheimer (MA) est la forme de démence la plus répandue dans le monde (43,8 millions de personnes atteintes en 2016) et en conséquence du vieillissement rapide de la population, l'incidence et l'impact socioéconomique de cette maladie devraient augmenter considérablement d'ici 2050 (100 millions de personnes seraient touchées). L'accumulation de peptides d'amyloïde bêta (Aβ) dans le cerveau, surtout les oligomères de Aβ toxiques, entraînent des lésions neuronales ainsi q'une perte synaptique. Ceci est un événement critique dans la pathogenèse de la MA. L'accumulation accrue de peptides Aβ peut se produire de deux manières: (1) la surproduction de Aβ à partir du clivage enzymatique de la protéine précurseur de l'amyloïde (APP) ou (2) une diminution de la dégradation et de la clairance des peptides Aβ. La clusterine est un chaperon extracellulaire lié à la MA sporadique par le biais d'études d'association pangénomique. Cependant,son rôle dans la pathogenèse de la MA n'est pas encore résolu. Par conséquent, ma thèse visait à examiner les effets de la clusterine sur la production de peptides Aβ par rapport à la dégradation. Les expériences de surexpression de la clusterine ont entraîné une augmentation des niveaux de peptides Aβ, un effet indépendant de la production de Aβ comme la clusterine n'a pas modulé les enzymes bêta ou gamma-sécrétase qui clivent en séquence l'APP. Ainsi, les données indiquent que la clusterine module probablement la dégradation et la clairance des peptides Aβ. Plus d'études sont nécessaires pour déterminer le mécanisme précis par lequel la clusterine peut augmenter les niveaux de peptides Aβ. Si l'impact négatif de la clusterine sur la dégradation et la clairance de l'amyloïde peut être contourné, cela fournirait une nouvelle stratégie thérapeutique pour lutter contre la pathogenèse de la MA.

2. Introduction

2.1 Alzheimer disease

Alzheimer disease (AD) was first described in 1906 by Dr. Alois Alzheimer in a lecture where he presented his patient Auguste Deter. Alzheimer described Deter as having rapidly increasing memory impairments, disorientation, perception disorders and many more symptoms [1]. Deter is now remembered as the first documented patient with AD, a term later coined by Emil Kraepelin in 1910 [2]. AD is now the world's most common form of dementia with an estimated 43.8 million affected individuals in 2016 and a predicted 100 million by 2050 [3, 4] and mainly affects elderly individuals. In the United States, 10% of people aged 65 and older are diagnosed with AD and this prevalence increases to 32% of people aged 85 and older [5]. AD accounts for approximately 60-70% of all dementia cases [6]. For comparison, the second most common dementia, vascular dementia, only accounts for 20-25% of total dementia cases [7]. Therefore, to address the global issue with the increasing incidence of dementia, it is obvious that understanding AD is of critical importance.

2.1.1 Alzheimer disease pathology

Amyloid plaques have been found in post-mortem brains of the majority of patients diagnosed with AD [8]. They are mainly composed of amyloid-β (Aβ) peptides [9] which are generated by the sequential enzymatic cleavage of Amyloid Precursor Protein (APP) by the beta- (BACE1) and gamma-secretase enzymes. The resulting Aβ peptides are of varying lengths such that the 40 amino acid species (Aβ40) is the most abundant, and the 42 amino acid species (Aβ42) is suspected as the principal culprit in AD pathology [10]. The deposition of Aβ during AD progression follows a general pattern, starting in the basal neocortex, continuing to the hippocampus, and lastly spreading to the cortex [8]. Aβ plaques were initially thought to be causative of neuronal toxicity, but more recently it has been proposed that it is the soluble oligomeric forms of Aβ, rather than Aβ monomers or insoluble fibrils, that cause loss of synaptic function [11]. Since amyloid plaques can be detected in cognitively normal individuals, this observation has indicated that plaque load is not the root cause of neurodegeneration [12]. Alternatively, intracerebroventricular

injections of soluble Aβ oligomers (dimers and trimers) in rats have specifically caused decreases in cognitive function and impairments to memory function [13]. In animal models of AD, soluble Aβ oligomers have been shown to inhibit hippocampal long-term potentiation (LTP) which is accompanied by loss of memory and decline in learning ability in these animals [14]. These findings implicate soluble Aβ oligomers as the toxic form of Aβ in the pathogenesis of AD.

2.1.2 APP processing

APP is processed through two distinct pathways. In non-amyloidogenic processing, APP is first cleaved by α-secretase to generate an 83 amino acid C-terminal fragment (APP-C83), which becomes a substrate for γ-secretase cleavage, producing APP Intracellular Domain (AICD) and a secreted fragment, p3 (Figure 1A). As the name implies, non-amyloidogenic processing of APP does not generate any Aβ peptides as the α-secretase cleavage site is within the Aβ sequence. In amyloidogenic processing, the first cleavage of APP is performed by Beta-Site APP-Cleaving Enzyme 1 (BACE1, β-secretase), producing APP-C99, followed by γ-secretase cleavage releasing Aβ peptides and AICD (Figure 1B) [15]. The sequential cleavage of APP by BACE1 then γ-secretase releases Aβ peptides of varying lengths, into the extracellular space [15]. γ-secretase is a protein complex that is composed of 4 subunits, among which the catalytic subunit is presenilin. There are two types of presenilin, encoded by separate genes, that can be integrated into a functional γ-secretase complex. These two presenilins are denoted simply as presenilin 1 (PS1) and presenilin 2 (PS2). Where the different presenilin-containing γ-secretases (PS1-γ-secretase or PS2-γ-secretase) differ is in their subcellular localization. PS1-γ-secretase is mainly located on the plasma membrane, whereas PS2-γ-secretase is confined to endosomes and lysosomes [16, 17].

While the initiating event leading to AD is still unknown, it is hypothesised that in AD there is a change occurring that increases Aβ production and/or decreases Aβ degradation with the net result of increasing Aβ levels in the brain. Aβ production is linked to APP metabolism and Aβ levels can be modulated by the respective involvement of either the

amyloidogenic or non-amyloidogenic pathway. Whether APP undergoes amyloidogenic or non-amyloidogenic processing is determined by several factors. Under physiological conditions, the majority of APP is processed in the non-amyloidogenic pathway, with the initial cleavage of APP being carried out by α-secretase [15]. α-secretase is located primarily at the plasma membrane and newly synthesised APP is transported from the trans-Golgi network to the plasma membrane [15, 18]. At the cell surface, APP is quickly cleaved by α-secretase followed by γ-secretase in the non-amyloidogenic pathway. The minority of APP that is not cleaved by α-secretase is re-internalized in clathrin-coated pits into an intracellular compartment where BACE1 is able to process APP in the amyloidogenic pathway. The distinct localization of α-secretase on the plasma membrane and BACE1 in intracellular compartments, allows for controlled regulation of APP processing by modulating the intracellular trafficking of APP. Intracellular trafficking of APP is largely controlled by post-translational modifications (PTMs) to the protein. APP can be subject to glycosylation, phosphorylation, palmitoylation, ubiquitination sumoylation, and sufation [18], all of which affect the localization of APP within the cell. There are several reports of differences in APP PTMs between AD brains and heathy controls that increase Aβ production [18], but it is still unclear if these PTMs are an initiating factor of AD or occur later in disease progression. It has also been reported that BACE1 protein levels and activity is increased in AD brains [19] while α-secretase activity decreases in AD [20, 21]. This suggests that changes in APP processing pathways could also be a contributing factor to AD pathogenesis.

Of the numerous Aβ species generated from the amyloidogenic processing of APP, Aβ40 and Aβ42 have been the most extensively studied since Aβ40 is the most abundant Aβ species and Aβ42 is suspected as the main culprit in the pathogenesis of AD [22]. As key biomarkers to stage the disease, changes to the Aβ40:Aβ42 ratio can differentiate between non-demented, non-Alzheimer dementia, and AD patients [22-24]. Recently, the Multhaup laboratory identified a novel enzymatic activity for BACE1 [25] whereby it can cleave larger Aβ species (e.g. Aβ40, Aβ42) into a common non-aggregating, non-toxic intermediate known as Aβ34 (Figure 1B). In their 2019 publication, measuring Aβ peptides related to both amyloid production (i.e. Aβ40, Aβ42) and degradation/clearance

(i.e. Aβ34) provided novel insights about the clinical staging of AD. By utilizing the Aβ34:Aβ42 ratio from cerebrospinal fluid (CSF) it is possible to identify patients in a pre-symptomatic phase, where no clinical symptoms are present, but have an elevated Aβ34:Aβ42 ratio. In other words, Aβ34:Aβ42 serves a better diagnostic accuracy for prodromal AD than Aβ40:Aβ42 ratio. Moreover, it was identified that BACE1 is a major Aβ degrading enzyme and its degradation product, Aβ34, is a major marker of total Aβ clearance, whose CSF levels show a strong correlation with overall Aβ clearance in amyloid plaque positive individuals.

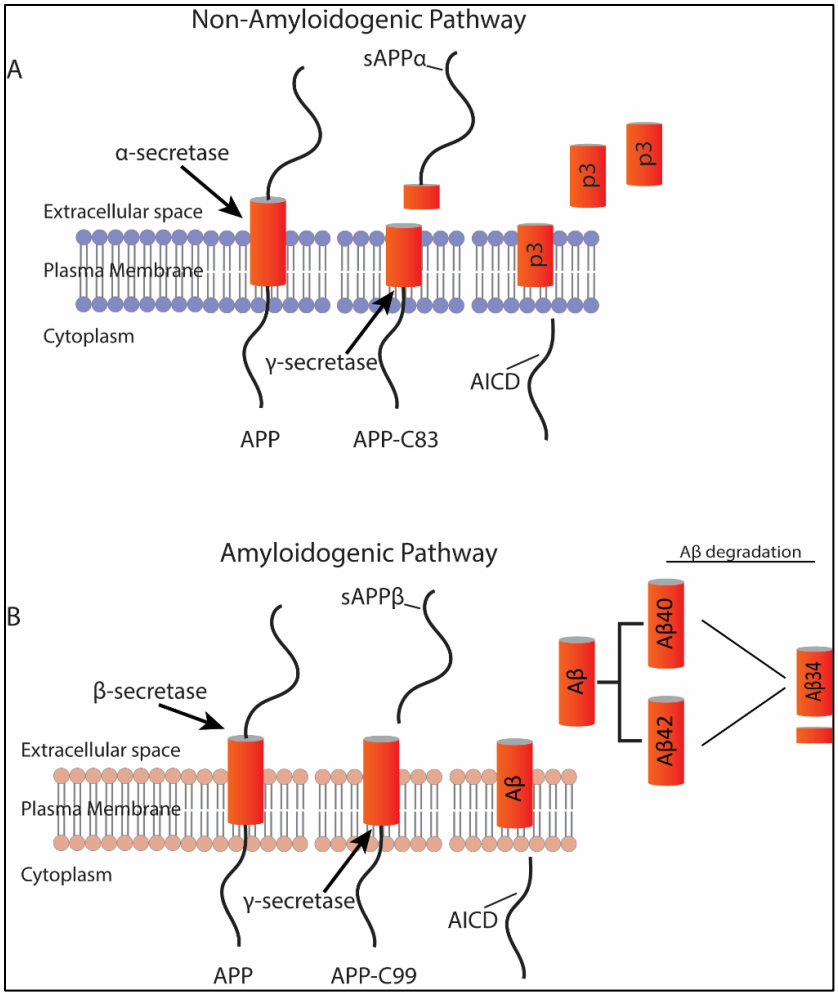

Figure 1: Amyloid Precursor Protein (APP) processing. (A) Non-amyloidogenic processing of APP by α-secretase, generating a soluble-APPα (sAPPα) fragment and the membrane bound C-terminal fragment (APP-C83). γ-secretase cleavage of APP-C83 releases the p3 fragment into the extracellular space and APP Intracellular Domain (AICD) into the cell. (B) Amyloidogenic processing of APP by β-secretase, generating soluble APPβ (sAPPβ) and the C-terminal fragment (APP-C99, APP-CTF). γ-secretase cleavage of APP-C99 generates AICD as well as Aβ peptides of varying lengths such as Aβ40 and Aβ42. Newly defined BACE1 activity results in cleavage of Aβ40 or Aβ42 into common Aβ34. Aβ sequence of APP is denoted in orange.

2.1.3 The amyloid hypothesis

Aβ peptides were discovered as the main constituents of senile plaques in 1985 [9] and this began the contemporary approach of AD research, which concentrates on understanding the pathological role of Aβ in AD [11]. Shortly after the discovery of Aβ, the

gene encoding the precursor protein from which Aβ peptides are derived was discovered and cloned [26]. This gene, aptly named *amyloid precursor protein* (APP), is located on chromosome 21. Before the *APP* gene was discovered and found to be somehow linked to AD, there were several reports that Down syndrome (DS) patients often had AD neuropathology. This information, combined with the identification of the *APP* gene located on chromosome 21, led to the hypothesis that Aβ accumulation from APP cleavage is the primary event in AD pathology [11].

Since DS patients have an extra copy of chromosome 21, which contains the *APP* gene, they would have higher APP protein levels and thus higher Aβ levels. Further evidence that it is the gene dosage of *APP* specifically that leads to AD pathology in DS patients comes from the identification of small cohort who have only a partial duplication of chromosome 21 that does not include *APP*. When there is only a duplication of the distal part of the chromosome, and no extra copy of *APP*, there is no observed AD pathology or symptoms [27]. The opposite partial chromosome 21 duplication has also been observed where patients have a duplication of the *APP* locus but do not have a full duplication of chromosome 21. These patients, like DS patients, present with early onset AD due to an increased level of APP protein leading to increased Aβ production [28].

Besides *APP* gene dosage, there are several mutations in *APP* that result in an increase in Aβ production which causes AD. These mutations cluster near to the BACE1 cleavage site in APP that increase its cleavage by BACE1, and consequently increases Aβ peptide production. For example, the APP K670N/M671L mutant (named the Swedish mutation), occurs at the BACE1 cleavage site and results in a large increase in Aβ peptide production and a familial form of AD [29]. In 2012, a unique cohort was discovered to have a decreased risk of cognitive decline in aging as well as a decreased incidence of AD in the elderly [30]. They were found to have the APP mutation A673T which reduces sAPPβ levels as well as Aβ production *in vitro* [30, 31]. The discovery of this protective mutation further strengthens the rationale of the amyloid hypothesis and emphasises the

importance of targeting both BACE1 and Aβ production in the prevention and treatment of AD.

2.2 Clusterin

Clusterin is a secreted glycoprotein with nearly ubiquitous expression by all mammalian cell types [32] and is a major component of physiological fluids including: plasma, milk, urine, cerebrospinal fluid and semen [33]. Clusterin was first discovered in ram rete testis fluid in 1983 as a major glycoprotein secreted by Sertoli cells [34]. Homologs of clusterin have been identified in all mammals with a sequence conservation of 70-80% between species [32].

2.2.1 Biology of clusterin

In humans, clusterin is encoded by the *CLU* gene located on the p-arm of chromosome 8 at position 8p21-p12 [32]. The *CLU* gene contains 9 exons and is transcribed in a single transcript encoding a 449 amino acid long protein. The pre-pro-peptide contains an N-terminal 22 amino acid long signal peptide, which traffics the protein to the lumen of the endoplasmic reticulum (ER) [32]. Once the pre-pro-peptide enters the ER, the signal peptide is removed followed by the addition of mannose-rich glycans to six asparagine residues and the creation of five intramolecular disulfide bonds, generating the aptly named pre-secretory clusterin (psClu) [35]. psClu is then trafficked to the trans-Golgi network where it is further glycosylated to a final carbohydrate content of approximately 25%; however, the extent of glycosylation is highly variable, fluctuating mainly between 17% and 27% of the total mass of the protein [36]. With the addition of complex carbohydrate molecules in the trans-Golgi network, the resulting protein is approximately 80 kDa in size. The final step in clusterin protein maturation is its cleavage into the N-terminal α-chain and the C-terminal β-chain. This cleavage event is performed by a furin-like proprotein convertase enzyme between amino acid residues 227 and 228 [37]. The resulting heterodimeric protein has the α and β chains oriented in an antisense manner and is released into the extracellular space through secretory vessels [33].

A 3D crystal structure of clusterin has yet to be determined and only limited experimental data exist regarding its conformation. Computational analyses of clusterin's amino acid sequence predicts that it contains three molten globule-like regions, which are natively disordered and sensitive to tryptic digestion [38]. UV circular dichroism spectroscopic analyses of clusterin revealed that its secondary structure is composed of approximately 62% α-helix, 8% β-sheet, 14% β-turn and 16% disordered regions at 25℃ [39]. The disordered regions presumably allow clusterin to bind to a wide variety of hydrophobic substrates, an integral property that mediates its ability to prevent protein aggregation [40, 41].

Expression of clusterin is influenced by several different stress conditions including: oncogene expression, growth factors and cytokines, chemotherapeutic drugs and several apoptosis- or stress-inducing conditions (heat shock, ultraviolet radiation, reactive oxygen species, etc…) [32]. The *CLU* promoter sequence is highly conserved through evolution and contains several stress-related transcription factor binding sites which are responsible for mediating the increase in clusterin expression under different stress conditions. Transcription factor binding sites in the *CLU* promoter region include: activator protein 1 and 2 (AP1 and AP2), heat shock element (HSE), cAMP response element (CRE), stimulatory element (SP1) and glucocorticoid response element (GRE) [32, 40].

Secreted clusterin (sClu) represents the main form of transcribed and translated clusterin, but is not the only isoform of the *CLU* gene. There are other variants of clusterin, including a nuclear form (nClu) and an intracellular form [37] which arise from alternative splicing under stress conditions. nClu has exon 2 removed resulting in a loss of the ER signal peptide and the translated protein is translocated to the cytoplasm and later to the nucleus. Unlike sClu, nClu is non-glycosylated and is a non-cleaved isoform with a molecular weight of 49kDa. nClu has 2 functional nuclear localization signals (NLSs) which allow it to translocate to the nucleus where it affects gene transcription through interactions with various transcription factors, ultimately resulting in the activation of cellular apoptosis [42]. There also exist several cytoplasmic isoforms of clusterin which

are similarly generated from alternative splicing of *CLU* under stress conditions. These cytoplasmic clusterin variants, unlike nClu, have been shown to not be involved in cellular apoptosis and collectively only constitute approximately 0.3% of the total clusterin mRNA [43]. Although the exact function of cytoplasmic clusterin is still not known, it has been proposed that it can affect intracellular signaling pathways, such as NF-κB signaling, under stress conditions [44].

2.2.2 Clusterin expression in the central nervous system

Clusterin expression is ubiquitously expressed by mammalian cells, however, cerebral clusterin mRNA and protein levels have been reported to be among the highest of all tissues [45]. *In-situ* hybridization and immunohistochemical analysis of rat brains identified that clusterin is highly expressed in hippocampal and cortical astrocytes [46]. While clusterin is expressed by all neuronal cell type to a certain extent, with the exception of microglia, clusterin expression in the brain has both regional and cell type differences. Neurons and astrocytes are reported as having the highest protein expression whereas microglia have no detectible clusterin expression [47, 48]. Clusterin mRNA levels in astrocytes appear to be similar in all brain regions of the rat brain. Contrastingly, neurons show distinct regional differences in their clusterin mRNA levels with the hippocampus, cerebral cortex and certain regions of the midbrain and cerebellum displaying clusterin mRNA while the striatum is completely lacking clusterin [47].

2.2.3 Clusterin in health and disease

Clusterin is a multifunctional protein which plays a role in diverse physiological functions including sperm maturation [49], lipid trafficking [50], inhibition of complement factors [51, 52], extracellular removal of aggregated proteins [53], inhibition of cellular apoptosis [54], regulation of cell cycle [55], protection against heat and oxidation-induced cellular stress [52, 56], and trafficking of cargo across the blood brain barrier (BBB) [57]. In addition to the roles of sClu listed above, there are several physiological functions of intracellular clusterin and nClu. nClu, for instance, has been reported to have a pro-apoptotic effect on cells under various stress conditions [42, 58]. Clusterin contains three large, flexible,

and intrinsically disordered regions, generally referred to as molten globule domains. These domains are commonly protein-protein interacting domains [59].

Despite the seemingly wide variety of functions that clusterin has, homozygous clusterin knockout in mice has no obvious deleterious effects. $Clu^{-/-}$ mice are viable, of normal size, and maintain their fertility. The only significant phenotype that has been reported was increased aggression in 50% of female $Clu^{-/-}$ mice, according to the International Mouse Phenotyping Consortium (IMPC) [60]. In a longer-term study, it was concluded that clusterin is not an essential protein for viability and survival as a complete knockout is not lethal. Mice lacking clusterin display glomerulopathy at 21 months of age indicating that clusterin has a protective role against age related kidney damage [61]. Clusterin knockout has also been reported to result in increased inflammatory response and inflammatory lesions with induced myocarditis in mice [62]. In a murine prostate cancer model, loss of clusterin increased metastatic spread of prostate cancer cells, and, in females of this mouse model which are normally cancer free, clusterin knockout increases tumorigenesis [63].

2.2.4 Role of clusterin in Alzheimer disease

Clusterin is reported to be a stress response protein in a wide variety of diseases from diabetes to cancer [32]. Clusterin has also been implicated in AD where its expression is increased in the brains of AD patients [64-66] and clusterin has been detected in the senile plaques of AD patients [66]. More recently, single nucleotide polymorphisms in the clusterin gene were associated with an increased risk of AD through genome wide association studies [67]. Since this discovery of clusterin's involvement in AD, many studies have tried to decipher how clusterin contributes to AD pathology, including its potential effects on the aggregation and/or clearance of Aβ peptides as well as cerebral amyloid angiopathy (CAA).

Clusterin's propensity to bind to extracellular aggregating proteins, and its presence in extracellular Aβ plaques, initially led to the conclusion that clusterin interacts with Aβ peptides and affects their deposition [66]. Evidence to support this idea was based upon the observation that AD-mediated increases in level of clusterin protein occurs most strongly in brain regions with high plaque load, mainly the hippocampus and frontal cortex [66]. Biochemical analyses showed that clusterin can inhibit the aggregation and fibril formation of synthetic Aβ42 peptide *in vitro* [68-70]. *In vitro* toxicity testing revealed that the addition of clusterin to synthetic Aβ42 reduced Aβ induced toxicity on cultured cells [69]. It was also shown that intraventricular injections of clusterin in 5xFAD mice (K670N/M671L, I716V, and V717I mutations in APP, and the M146L and L286V mutations in presenilin 1) reduced cognitive decline, reduced the severity of cerebral amyloid angiopathy, and reduced the levels of soluble Aβ40 and Aβ42 [71]. Overall, these findings suggested that clusterin plays a neuroprotective role in AD, possibly through neuronal cells increasing clusterin expression and secretion to combat against Aβ aggregation and deposition, reducing neuronal toxicity and thus slowing disease progression.

In contrast to these findings, clusterin knockout mice crossed with APP transgenic mice decreased the amount of fibrillar Aβ in the brains of the transgenic mice [72]. Adding to the potentially diverse roles of clusterin in AD, it was shown that clusterin can either promote or prevent the formation of amyloid fibrils, depending on the clusterin to substrate ratio. A low clusterin to substrate ratio results in increased fibril formation, whereas a high ratio has the opposite effect and slows fibril formation [69].

There is also evidence suggesting that clusterin plays a role in clearance by trafficking Aβ peptides across the blood brain barrier (BBB) through receptor mediated transport [57]. *In vivo* studies identified that clusterin-Aβ complexes are transported across the BBB at a faster rate than either Aβ or clusterin alone. This demonstrates that there is an increased clearance of Aβ peptides from the brain to the vasculature when Aβ is in complex with clusterin [57]. For example, clusterin forms a complex with Aβ40 *in vitro* and

this complex has a strong affinity for Lipoprotein receptor-related protein 2 (LRP2), a major clusterin transporter [73]. Aβ40 did not bind to LRP2 alone, but when Aβ40-clusterin preformed complexes were analysed, they had a strong affinity for LRP2 which resulted in increased cellular uptake of Aβ40 [73].

Clusterin is secreted in complex with cholesterol by astrocytes and is transported to neurons and taken up by cholesterol transporters. Clusterin transport of cholesterol to neurons can potentially affect APP processing indirectly by affecting the cholesterol content in neurons, which has been identified to affect APP processing and Aβ release [74]. It was found that clusterin transport of cholesterol to neurons and endothelial cells forming the BBB can alter the cellular expression levels of APP, as well as the non-amyloidogenic enzyme ADAM10 and the amyloidogenic enzyme BACE1. Lastly, clusterin can affect the uptake of extracellular Aβ peptides by endothelial cells of the BBB thus reducing the efflux of Aβ from the brain to the peripheral circulation [74].

Overall, clusterin's involvement appears to be a response to pre-existing amyloid burden and not an initiating factor in AD pathogenesis. Clusterin levels are increased in AD and clusterin expression increases in response high cerebral Aβ levels. Additionally, the majority of experimental evidence suggests that this increase is a protective response rather than detrimental, by preventing cognitive decline and neuronal damage in AD animal models.

In addition to investigating clusterin's role in AD pathology, emerging literature is focused upon the potential of clusterin as a biomarker of AD [60, 75-77]. Compared to other CSF-based biomarkers, such as Aβ40 and Aβ42, which require patients to undergo invasive and costly lumbar punctures, clusterin is an attractive alternative because it could be readily analyzed from a simple blood sample. Notably, clusterin levels in plasma are strongly correlated with increased atrophy in the hippocampus and disease severity in AD patients [75, 78]. Clusterin is significantly increased at both the mRNA and protein level

in AD patients compared to those with mild cognitive impairment (MCI) and there was a strong negative correlation between plasma clusterin levels and cognitive impairment [75]. Other studies have also determined that the post-translational state of clusterin (e.g. glycosylation of asparagine 64 within the α-chain) can predict the level of hippocampal atrophy in AD patients [76]. Overall, these studies demonstrate the promising potential for clusterin as a non-invasive AD biomarker.

One of the major identified functions of clusterin in the CNS is as an ATP-independent non-folding chaperone protein, a so-called “holdase” protein [41]. Considering also that clusterin has been found as a constituent of amyloid plaques from post-mortem AD patient brains [66], I hypothesise that clusterin is influencing AD pathology by regulating the production and/or clearance of Aβ peptides. Therefore, my thesis has investigated the ability of clusterin to modulate Aβ peptide levels in a variety of cell-based assays.

3. Results

3.1 Clusterin overexpression increase Aβ40 levels in a dose dependent manner

To assess the effect of clusterin on Aβ abundance, I began by using a Human Embryonic Kidney (HEK) cell system, where I transiently transfected cells with clusterin and/or APP. APP was overexpressed to have an excess of precursor protein for large production of Aβ peptides. Cell media and lysates were analysed by SDS-PAGE/Western blotting to confirm the overexpression of proteins (Figure. 2 A-B) Conditioned media from these cells was collected and analyzed via a sandwich-based enzyme-linked immunosorbent assay (ELISA) developed in the Multhaup laboratory to specifically quantify Aβ40, Aβ42 and Aβ34 levels (Figure. 2 C-E). I found that in the media from cells that overexpressed both clusterin and APP, there was a specific trend towards an increase in Aβ40 levels, but no effect to Aβ42 or Aβ34.

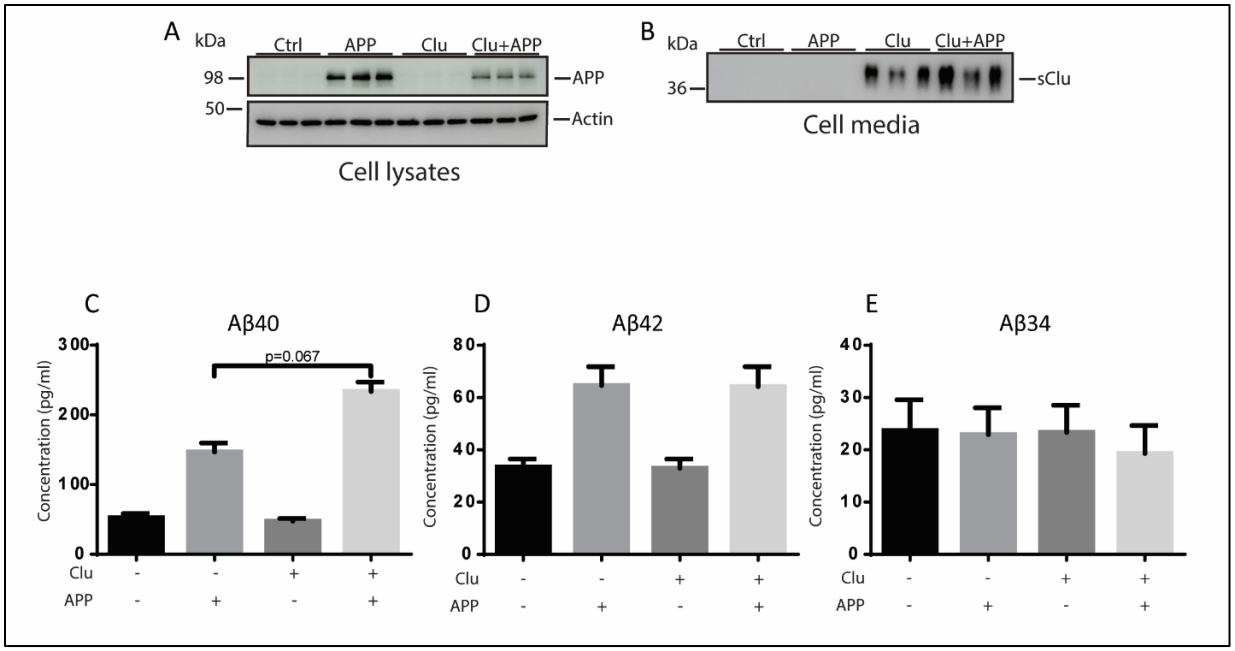

Figure 2: **Clusterin-APP co-expression leads to increased Aβ40 levels.** HEK293 cells were transiently transfected with APP, clusterin or both. Conditioned cell media and cell lysates were harvested after 24h for analysis. (A-B) Western blot analysis of APP and clusterin (sClu) expression, from cell lysates (A) and conditioned media (B), respectively. (C-E) Sandwich-based ELISA analysis of Aβ40 (C), Aβ42 (D), and Aβ34 (E) from conditioned media. Aβ levels are displayed as concentration (pg/ml). Statistical analysis performed using a Student's T-test, error bars indicate the mean ±SEM, n=3.

Following this initial result, titration experiments were performed in which the overexpression of APP was held constant but there were increasing levels of clusterin overexpression, as confirmed by Western blot analyses of the conditioned media (Figure 2B). I used HEK-293 cells and transiently transfected them, conditioned the media and collected for analysis by sandwich-based ELISA (Figure. 3 C-E). In agreement with the preliminary data, there was a gradual increase in Aβ40 levels with increasing clusterin overexpression, whereas there was no significant difference in Aβ42 or Aβ34 levels across any of the conditions.

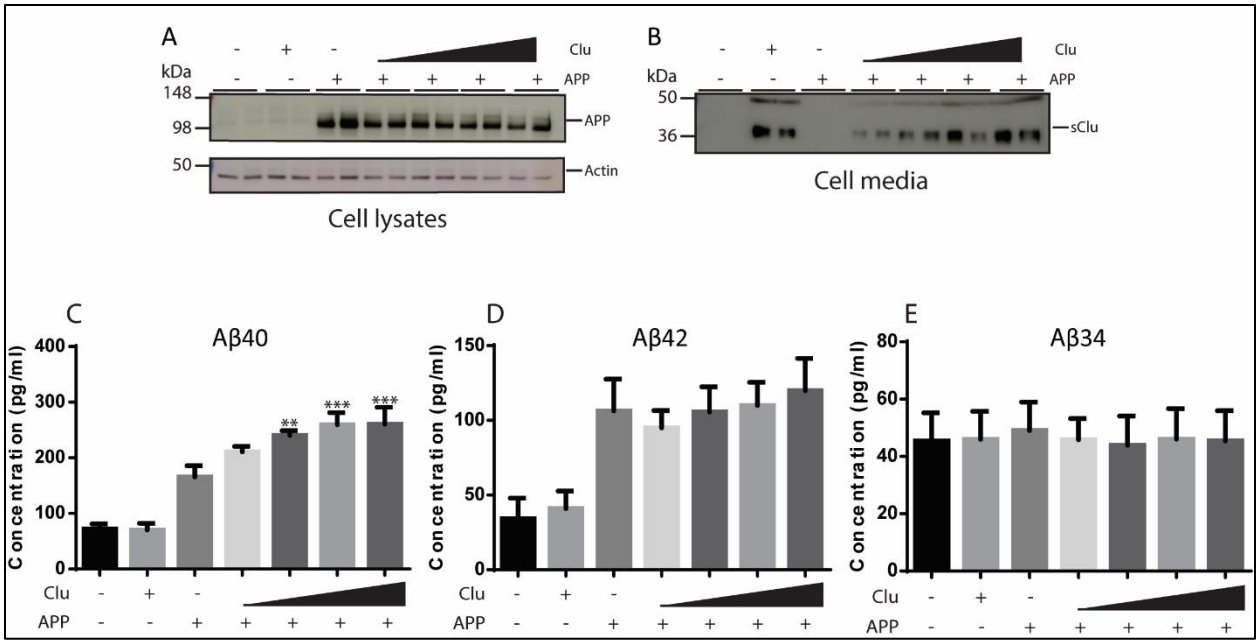

Figure 3: Increasing clusterin expression results in increased Aβ40 levels. HEK293 cells were transiently transfected with an increasing amount of clusterin while maintaining a constant amount of APP. Conditioned cell media and cell lysates were harvested after 24h for analysis. (A-B) Western blot analysis of APP and clusterin (sClu) expression, from cell lysates (A) and cell media (B), respectively. (C-E) ELISA analysis of Aβ40 (C), Aβ42 (D), and Aβ34 (E) from conditioned media. Aβ levels are displayed as concentration (pg/ml). Statistical analysis performed using one-way ANOVA followed by Dunnett's test, error bars indicate the mean ±SEM, n=3, **p<0.004, *** p<0.0007.

To cross-validate the HEK-293 cells findings, I proceeded to using a neuron-like cell type, SH-SY5Y. Clusterin was transiently transfected into two different SH-SY5Y cell lines (1) SH-SY5Y cells that stably overexpressed Mock plasmid and (2) SH-SY5Y cells that stably overexpressed APP. Cell lysates and conditioned media were analysed by Western blotting to confirm the proper protein overexpression (Figure. 4 A-B) and the conditioned cell media were analysed by sandwich-based ELISA to quantify Aβ levels (Figure. 4 C-H). Contrary to the initial result in HEK-293 cells, in both SH-SY5Y cell types tested, there is a significant increase in both Aβ40 and Aβ42. These experiments have identified that clusterin has an effect that results in changes to Aβ levels in both HEK-293 cells and SH-SY5Y cells, with cell type differences in the effect. There are two possibilities for how clusterin is influencing Aβ levels: (1) by affecting Aβ production through affecting enzymatic activity of BACE1 and/or γ-secretase or (2) affecting clearance of Aβ peptides.

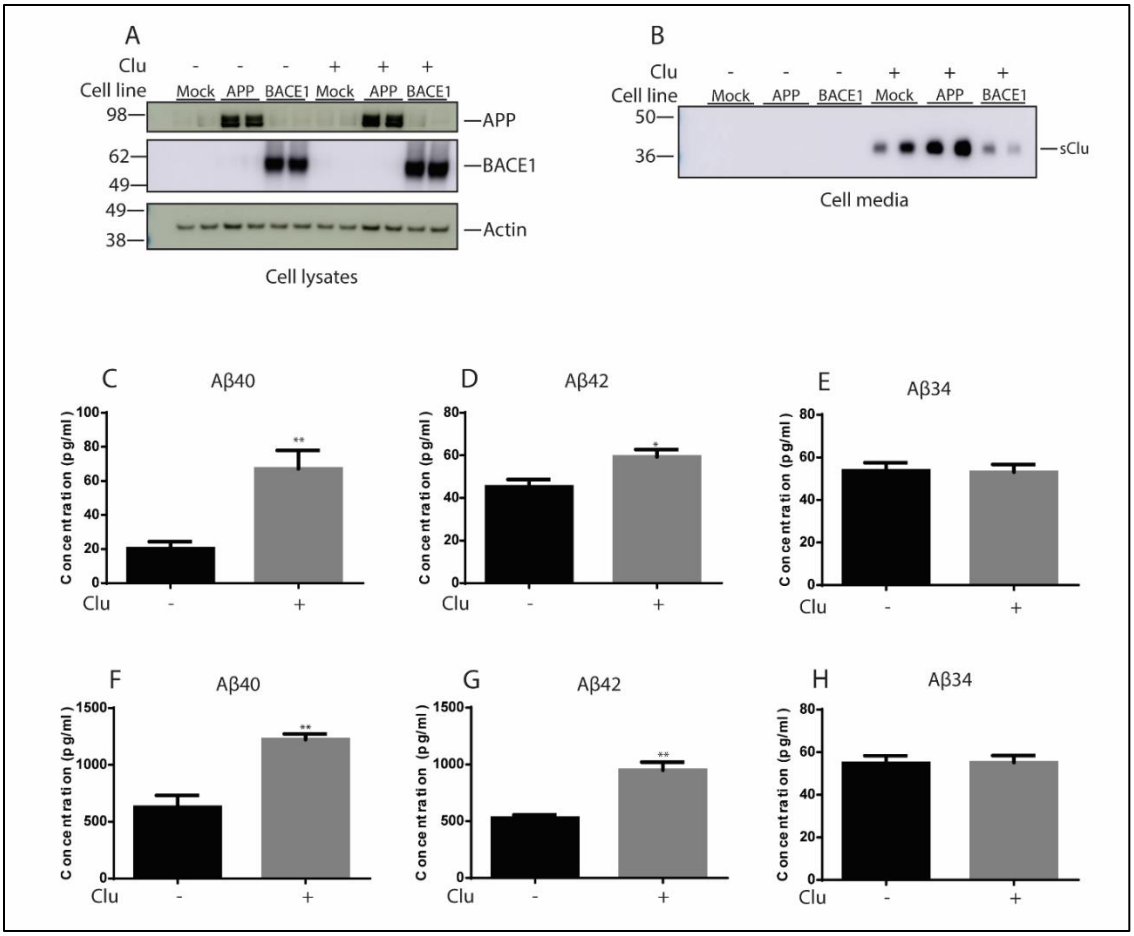

Figure 4: Clusterin overexpression in SH-SY5Y with endogenous or overexpressed levels of APP results in increased Aβ40 and Aβ42. SH-SY5Y cells stably overexpressing mock plasmid or APP were transiently transfected with a control or clusterin plasmid. Conditioned cell media and cell lysates were harvested after 24h for analysis. (A-B) Western blot analysis of BACE1, APP and clusterin expression from cell lysates (A) and cell media (B), respectively. (C-E) ELISA analysis of Aβ40 (C), Aβ42 (D), and Aβ34 (E) from mock plasmid-stably overexpressing SH-SY5Y conditioned cell media. (F-H) ELISA analysis of Aβ40 (F), Aβ42 (G), and Aβ34 (H) from APP-stably overexpressing SH-SY5Y conditioned cell media. Aβ levels are displayed as concentration (pg/ml). Statistical analysis performed using a Student's T-test, error bars indicate the mean ±SEM, n=6, * $p<0.05$, ** $p<0.01$.

3.2 BACE1 cleavage of APP is not affected by clusterin

If clusterin is affecting Aβ production, it could be exerting its effects on either BACE1 and/or on γ-secretase, the two enzymes involved in Aβ production. In the first scenario, clusterin overexpression results in increased levels of Aβ peptides by increasing the cleavage of APP by BACE1. To address this hypothesis, I used SH-SY5Y cells, stably

overexpressing BACE1 and transiently transfected these cells with clusterin. Conditioned cell media was collected and Aβ levels were quantified by sandwich-based ELISA (Figure. 5 C-E). As seen in the initial HEK-293 cell experiments, here I showed a significant increase in Aβ40 levels, with no significant change to Aβ42 or Aβ34 levels. When comparing BACE1 stably overexpressing cells with clusterin overexpression to those not overexpressing clusterin, there is no significant increase in Aβ34. The overall result using BACE1 stably overexpressing cells differs from the previous SH-SY5Y experiments (stably overexpressing mock plasmid or APP) where I reported an increase in both Aβ40 and Aβ42, here there was only a significant increase in Aβ40. In addition to being a critical enzyme in Aβ production [79], BACE1 is a major enzyme producing Aβ34 from longer Aβ species, such as Aβ40 and Aβ42 [80]; therefore, BACE1 overexpression results in a large increase in Aβ34. This Aβ cleaving activity of BACE1 can explain the why no significant increase in Aβ42 was observed with BACE1 overexpression. Overexpression of BACE1, which uses Aβ42 as a substrate for Aβ34 generation [80], would counteract the clusterin-mediated increase in Aβ42 levels by converting Aβ42 into Aβ34.

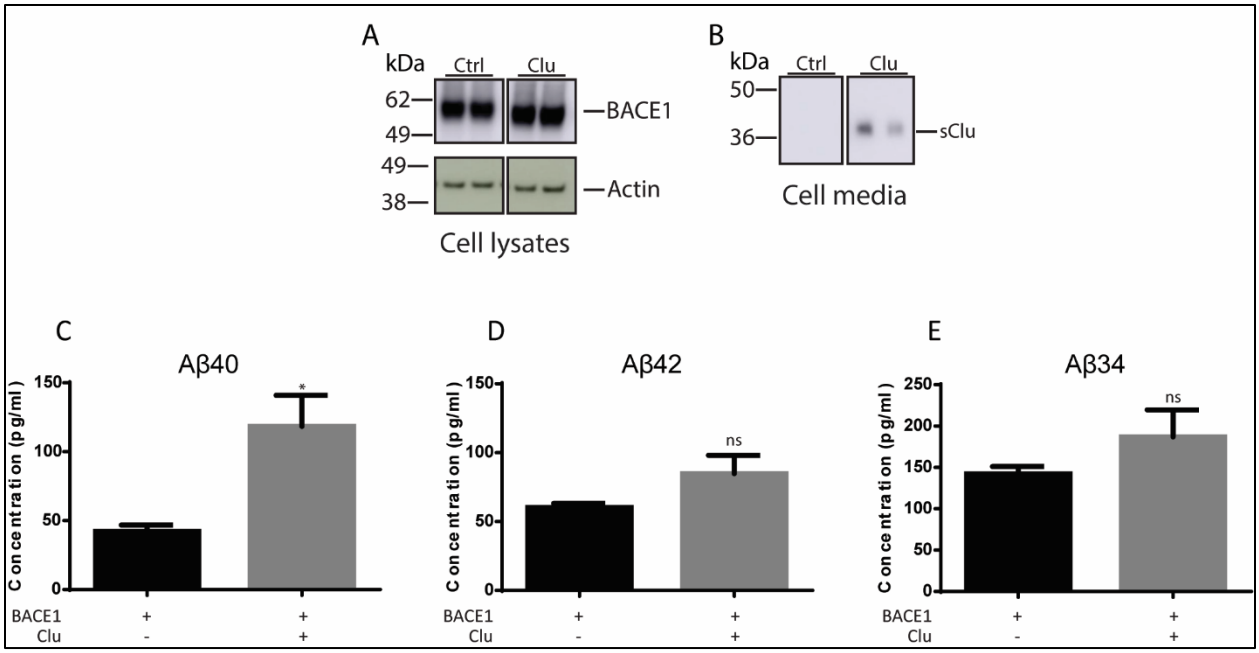

Figure 5: BACE1 overexpression does not affect clusterin-mediated increase in Aβ40 but prevents clusterin-mediated increase in Aβ42. SH-SY5Y cells stably overexpressing BACE1 were transiently transfected with a control or clusterin plasmid. Conditioned cell media and cell lysates were harvested after 24h for analysis. (A-B) Western blot analysis of BACE1 and clusterin expression from cell lysates (A) and cell media (B), respectively. (C-E) ELISA analysis of Aβ40 (C), Aβ42 (D), and Aβ34 (E) from conditioned cell media. Aβ levels are displayed as concentration (pg/ml). Statistical analysis performed using a Student's T-test, error bars indicate the mean ±SEM, n=3, * p=0.016, ns= non-significant change

3.3 γ-Secretase cleavage of APP-CTF is not affected by clusterin

To assess if clusterin is affecting γ-secretase activity causing an increase in Aβ40 from cleavage of APP C-terminal fragments (APP-CTFs), I performed siRNA mediated knockdown of the catalytic subunit of γ-secretase and determined if the clusterin-mediated increase of Aβ40 was still observed. Recall that PS1-γ-secretase is mainly located on the plasma membrane, whereas PS2-γ-secretase is confined to endosomes and lysosomes [16, 17]. Since PS1-γ-secretase is mainly at the plasma membrane and clusterin is a secreted protein, I hypothesise that if clusterin is affecting γ-secretase activity, then it will have an effect on PS1-γ-secretase and not PS2-γ-secretase because clusterin would have access to directly interact with PS1-γ-secretase and not PS2-γ-secretase. To test this hypothesis, I utilized individual knockdown of PS1 or PS2, in

combination with transiently overexpressed clusterin, and assessed if the effect of clusterin was altered.

Wild-type (WT) SH-SY5Y cells were transiently transfected with a clusterin plasmid, for overexpression, while simultaneously treated with siRNA against either PS1 or PS2. Cell media was conditioned and then collected alongside the cell lysates. The lysates were run on SDS-PAGE/Western blot to confirm the protein knockdown efficiency (Figure. 6 A-B), and the cell media were analyzed by sandwich-based ELISA to quantify the Aβ40, Aβ42 and Aβ34 levels (Figure. 6 C-E). Statistical analysis revealed that there was a significant effect of PS1 knockdown on Aβ40 and Aβ42 levels, but no significant interaction effect of clusterin overexpression and PS knockdown on any Aβ species tested. Wild-type SH-SY5Y cells do not have high enough levels of Aβ34 to be detected with our sandwich-based ELISA; therefore, to have detectable amounts of Aβ34, I performed the same experiment in BACE1-stably-overexpressing cells. Using BACE1-stably-overexpressing cells allows to identify if there is any effect of clusterin on BACE1's cleavage of longer Aβ peptides into Aβ34. In addition, this system enables us to assess if there is any effect of clusterin on Aβ34 levels, in a similar manner to the effect on Aβ40. In this experiment, it was seen that, similarly to the PS1/PS2 knockdown experiment performed in WT SH-SH-SY5Y, there was no significant interaction effect of clusterin overexpression and PS knockdown on Aβ levels (Figure. 7 C-E). There was no significant effect of clusterin on Aβ34 levels. This finding is consistent with the previous result obtained in BACE1-stably-overexpressing cells with transient clusterin overexpression, which did not show any effect of clusterin on Aβ34. There was a significant effect of PS2 knockdown on decreasing Aβ34 levels. Together, these results show there is no effect of clusterin on Aβ production under the experimental conditions tested, therefore, it is our hypothesis that clusterin is mediating its effect on Aβ levels by altering Aβ clearance.

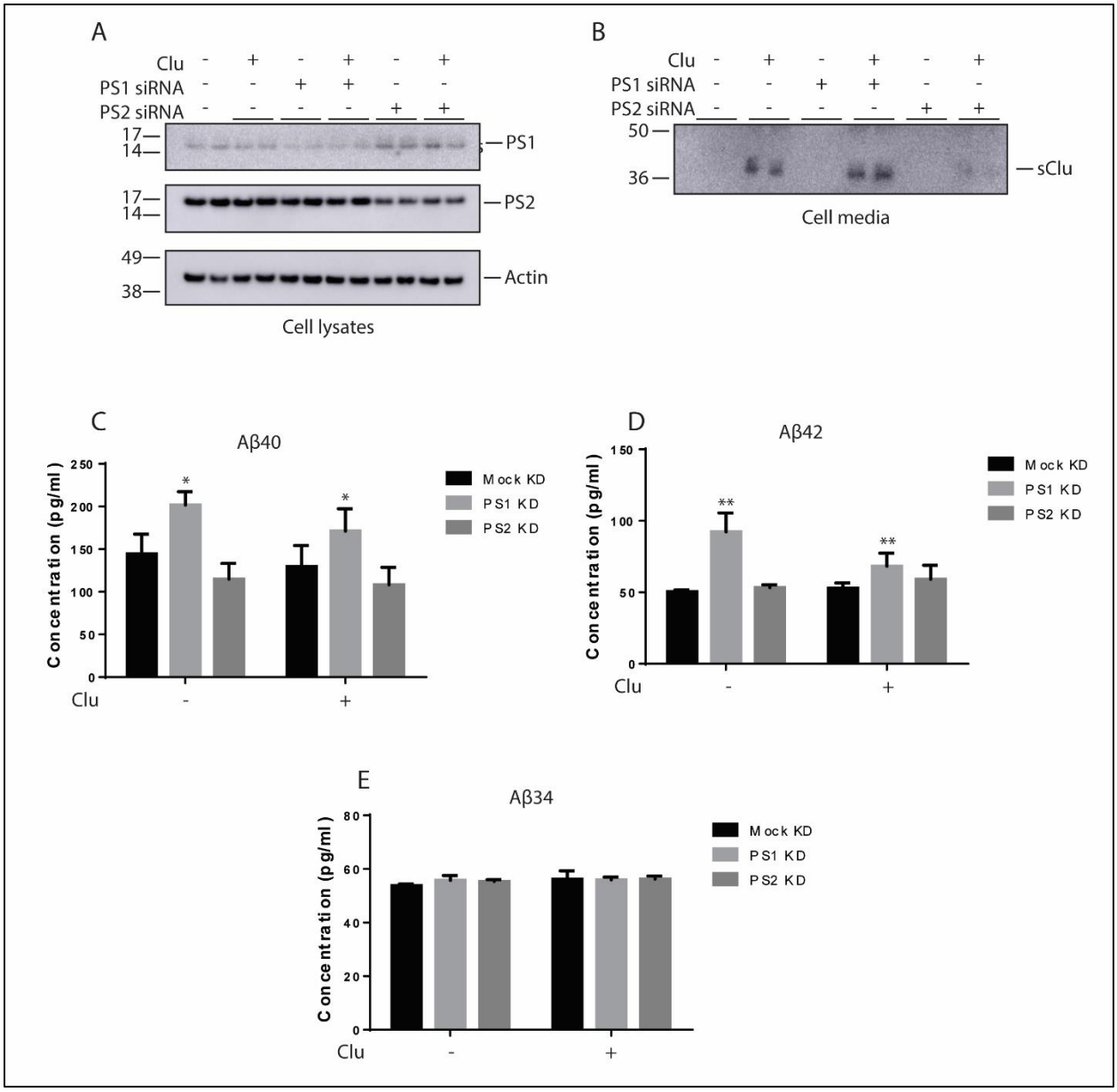

Figure 6: Clusterin-mediated increase in Aβ levels is not affected by siRNA-mediated knockdown of Presenilin 1 or Presenilin 2 in wild-type SH-SY5Y cells. SH-SY5Y cells were simultaneously transfected with a clusterin plasmid and siRNA against with Presenilin 1 or Presenilin 2. Conditioned cell media and cell lysates were harvested after 72h for analysis. (A-B) Western blot analysis of Presenilin knockdown and clusterin overexpression from cell lysates (A) and cell media (B), respectively. (C-E) ELISA analysis of Aβ40 (C), Aβ42 (D), and Aβ34 (E) from conditioned cell media. Aβ levels are displayed as concentration (pg/ml) Statistical analysis performed using two-way ANOVA without repeated measures. Error bars indicate mean ±SEM, n=3. No significant row or interaction factor calculated. Significant column factor identified, *p<0.05, **p<0.01.

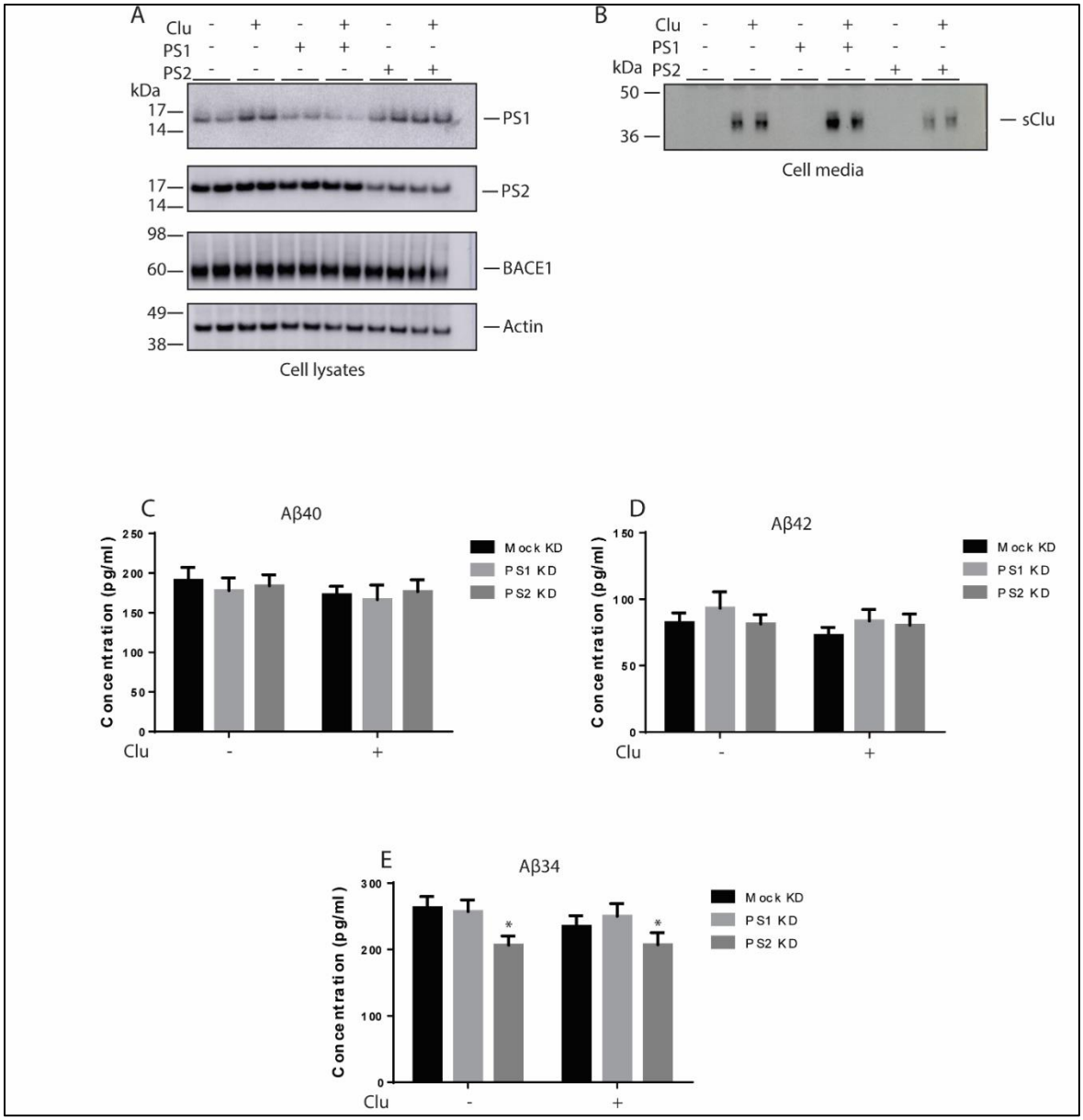

Figure 7: Clusterin-mediated increase in Aβ levels is not affected by siRNA-mediated knockdown of Presenilin 1 or Presenilin 2 in BACE1-stably overexpressing cells. BACE1-stably overexpressing SH-SY5Y cells were simultaneously transfected with a clusterin plasmid and siRNA against with Presenilin 1 or Presenilin 2. Conditioned cell media and cell lysates were harvested after 72h for analysis. (A-B) Western blot analysis of Presenilin knockdown and clusterin overexpression from cell lysates and media respectively. (C-E) ELISA analysis of Aβ40 (C), Aβ42 (D), and Aβ34 (E) from conditioned cell media. Aβ levels are displayed as concentration (pg/ml) Statistical analysis performed using two-way ANOVA without repeated measures. Error bars indicate mean ±SEM, n=6. No significant row or interaction factor calculated. Significant column factor identified, *p<0.05.

4. Discussion

Clusterin's involvement in AD pathology has remained a contentious subject in the field since the first report of its link to AD. Multiple groups have reported an increase in clusterin from analysis of post mortem brains of AD patients [65, 66, 81] as well as in AD mouse models [23, 74]. Many studies report that clusterin has a beneficial role in AD through inhibiting Aβ aggregation [68, 69, 72, 82-84], clearance of Aβ peptides [57, 72, 73] and preventing Aβ mediated toxicity [69, 71]. Opposingly, there are many groups who report that clusterin is non-neuroprotective or detrimental by mediating Aβ toxicity [85-87] and increasing Aβ deposition [69, 88]. In this thesis I aimed to investigate clusterin's influence on Aβ peptide levels by interrogating the effect of clusterin on Aβ peptide generation and degradation.

4.1 Clusterin overexpression increase Aβ40 levels in a dose dependent manner in HEK-293 cells

My investigations began by overexpressing APP together with clusterin in HEK-293 cells and measured the effect of clusterin overexpression on Aβ peptide levels, specifically Aβ40, Aβ42 and Aβ34. Aβ40 and Aβ42 were under investigation because it has long been known that Aβ40 is the most abundant Aβ species and Aβ42 is considered as the main pathological species in AD [22]. Aβ34 is of interest because of it has been reported to be useful as a biomarker of Aβ degradation [80].

My preliminary experiments showed that clusterin overexpression increases the levels of Aβ40 specifically in HEK-293 cells when APP is also overexpressed. Since the increase in Aβ40 was non-significant ($p=0.06$), I wanted to confirm that the observed effect was indeed truly clusterin-dependent. Therefore, to further validate this result, I performed a titration experiment in which I had increasing expression of clusterin with constant overexpression of APP and measured Aβ levels. In agreement with my initial experiment, this experiment showed an increase in Aβ40 levels had a dose dependent relationship with clusterin overexpression but had no effect on Aβ42 or Aβ34. From this dose-

dependent relationship between clusterin and Aβ40 levels, I was much more confident that the observed effect is clusterin-mediated.

4.2 Clusterin increases both Aβ40 and Aβ42 in SH-SY5Y cell systems

Following the experiments in HEK-293 cells, I decided to proceed to using SH-SY5Y human neuroblastoma cells as a model system. This cell line has the advantage of expressing higher endogenous levels of APP, compared to HEK-293 cells, resulting in higher levels of endogenous Aβ peptides that can be readily detected by ELISA. I decided to use both APP-stably overexpressing SH-SY5Y cells (SY5Y-APP) as well as mock plasmid-stably overexpressing cells (SY5Y-mock) to test if clusterin overexpression would have the same effect in SH-SY5Y cells as it did in HEK-293 cells. SY5Y-APP cells were used to compare to SY5Y-mock, to ensure that the effect seen with clusterin overexpression is not an artifact of overexpressing two proteins. In both SY5Y-APP as well as SY5Y-mock cells, there was a statistically significant increase in Aβ40 levels when clusterin was overexpressed. Since clusterin overexpression in both SY5Y-APP and SY5Y-mock cells had the same outcome, the data indicate that the effect of increasing Aβ40 was indeed clusterin-mediated and not a confounding effect of overexpressing two proteins simultaneously.

Unlike in the previous experiments using HEK-293 cells, clusterin overexpression in these SH-SY5Y model systems also led to a statistically significant increase in Aβ42. This observation goes against our previous hypothesis that clusterin was exerting an effect specifically on Aβ40. A possible explanation for the clusterin effect being extended to Aβ42 in SH-SY5Y cells compared to HEK-293 cells is the difference in relative Aβ40 to Aβ42 levels between these model systems. In the HEK-293 cell experiment, there was approximately three-fold more Aβ40 compared to Aβ42 when APP was overexpressed, whereas in both SY5Y-APP and SY5Y-mock cells, there is closer to equal amounts of Aβ40 and Aβ42. It is possible that the effect of clusterin on Aβ levels is occurring through direct interaction between clusterin and the Aβ peptide in question, which leads to the inhibition of the degradation of any bound Aβ species. These results would indicate that

clusterin is not specifically binding to Aβ40, but rather that it has a higher affinity for Aβ40 over Aβ42, and thus, has a primary and larger effect on Aβ40, likely decreasing its clearance.

The reason that no effect was seen for Aβ42 from the HEK-293 cell experiment is because Aβ42 levels were not high enough to compete with the abundance of Aβ40 in these cell media. If there is a large excess of Aβ40 compared to Aβ42, and if clusterin is mediating its effect through direct binding to Aβ peptides, then it is likely that no changes to Aβ42 were observed in the HEK-293 cell experiments because Aβ40 had saturated all the available clusterin, so there was no free clusterin left to bind Aβ42. The hypothesis that clusterin is exerting its effect through direct interaction is supported by research that measured the affinity of clusterin for Aβ40 and Aβ42 [68, 82]. In this study, the authors, Matsubara *et al.*, determined that clusterin's affinity for Aβ40 and Aβ42 are almost equivalent, with slightly higher affinity for Aβ42 over Aβ40 [68]. In contrast, my results would rather indicate that that clusterin has a higher affinity for Aβ40. This difference could be attributed to the difference in experimental conditions and the experimental endpoints. For instance, my experiments were performed in a cell expression system, whereas Matsubara *et al.* used an *in vitro* system with purified clusterin and synthetic Aβ peptides. However, the hypothesis that clusterin binding to Aβ peptides prevents their degradation is supported by research reporting that clusterin-Aβ40 or clusterin-Aβ42 complexes were resistant to proteolytic digestion by trypsin, chymotrypsin and plasmin [68]. Future experiments are required to confirm or refute this hypothesis.

4.3 BACE1 cleavage of APP is not affected by clusterin

The increase in the levels of Aβ peptides upon clusterin overexpression could be an effect of clusterin on production of Aβ peptides and/or their clearance. I thought it was necessary to determine if clusterin influenced the shedding of APP to promote the production of certain Aβ peptides. To this end, I began the investigation with BACE1, which is responsible for the generation of APP-C99 from APP. To assess if clusterin affected BACE1 cleavage of APP, I used a BACE1-stably overexpressing SH-SY5Y (SY5Y-

BACE1) cell line and transiently overexpressed clusterin. Here, I observed a significant increase in Aβ40 with no change to Aβ42 or Aβ34 upon clusterin overexpression. This result contrasts to the previous SH-SY5Y experiment where I observed a significant increase in both Aβ40 and Aβ42. A possible explanation for this result is the following; since BACE1 is overexpressed in this system, clusterin's effect on Aβ40 and Aβ42, i.e. increasing their levels, could be dampened by BACE1 cleavage of Aβ40 and Aβ42. The mechanism by which this process occurs comes from recent research by Liebsch *et al.* that identified that BACE1 cleaves longer Aβ species into Aβ34 [25]. The BACE1 cleavage of Aβ40 and Aβ42 into Aβ34 would decrease the levels of these Aβs and BACE1 overexpression in this system would effectively be working in opposition of clusterin's effect on Aβ levels.

As detailed above, I hypothesize that the Aβ cleaving activity of BACE1 is dampening clusterin's effect by reducing the increase in Aβ42 levels to the point that there is no longer a significant difference with clusterin overexpression. There is still a significant increase in Aβ40 which could be attributed to BACE1's substrate preference between different Aβ species or it could be credited to clusterin's distinct affinity for different Aβs. The larger increase in Aβ40 levels compared to Aβ42 in this experiment is in keeping with our hypothesis that the effect of clusterin has a stronger influence on Aβ40 than for Aβ42, possibly due to higher affinity for Aβ40 over Aβ42. Moreover, clusterin with BACE1 overexpression did not affect Aβ levels in a different manner than clusterin had alone or with APP overexpression. This means that BACE1 is not involved in the mechanism by which clusterin affects Aβ levels because overexpression of BACE1 did not change the overall effect of clusterin.

Additional insight that using the SY5Y-BACE1 cell line provides is the confirmation that there was no effect of clusterin on increasing Aβ34 levels despite there being a high abundance of this peptide in these cell media. From this, the data indicates that clusterin is not affecting Aβ34 in the same way that it affects Aβ40 and Aβ42. There was still a significant increase in Aβ40 despite there being a higher amount of Aβ34 over Aβ40. If

clusterin is mediating its effect through direct interaction with Aβ peptides, then I hypothesize that the affinity of clusterin for Aβ34 will be significantly lower than the affinity for Aβ40 or Aβ42 since high Aβ34 levels did not outcompete clusterin's effect on Aβ40. The results of this experiment indicate that clusterin does not alter BACE1 cleavage of APP nor does it affect BACE1 cleavage of longer Aβ species into Aβ34.

4.4 γ-Secretase cleavage of APP-CTF is not affected by clusterin

After determining that BACE1 cleavage of APP is not affected by clusterin, the next enzyme in the sequential cleavage of APP to Aβ to test is the γ-secretase complex. The γ-secretase complex can have either of two catalytic subunits: Presenilin 1 (PS1) or Presenilin 2 (PS2). Depending on which presenilin is included in the complex, the subcellular localization of the complex is different. Generally, PS2-γ-secretase is localized to endosomes and lysosomes, whereas PS1-γ-secretase is mainly localized on the plasma membrane [16, 17]. Considering these different sub-cellular localizations and that clusterin is a secreted protein present in the extracellular environment, I hypothesized that if clusterin is increasing Aβ40 and Aβ42 levels by affecting γ-secretase cleavage, then clusterin will affect PS1-γ-secretase cleavage because it would have direct access to interact with PS1-γ-secretase at the plasma membrane and not PS2-γ-secretase which is located inside the cell (Figure 8). To test if clusterin is having its effect on Aβ40 levels through affecting γ-secretase to produce more of these peptides, I used individual siRNA-mediated knockdown of PS1 or PS2 with clusterin overexpression. Consistent with the BACE1 experiment above, this experiment aims to identify if there is a change to the overall effect of clusterin on Aβ peptides, that has been observed in the previous experiments, by knockdown of either PS1 or PS2.

When analysing the conditioned media from wild-type SH-SY5Y cells with PS1 or PS2 knockdown there was a significant increase in Aβ40 and Aβ42 that corresponded to the PS1 knockdown, but there was no significant interaction factor identified between clusterin and either PS1 or PS2 knockdown. This indicates that there is no combinatorial effect of clusterin overexpression with either PS1 or PS2 knockdown; therefore, the data

indicate that the effect of clusterin on Aβ levels is not though a mechanism that affects γ-secretase cleavage of APP-C99 into different Aβ peptides. Overall, clusterin appears to be altering Aβ levels through mechanism that does not affect Aβ production.

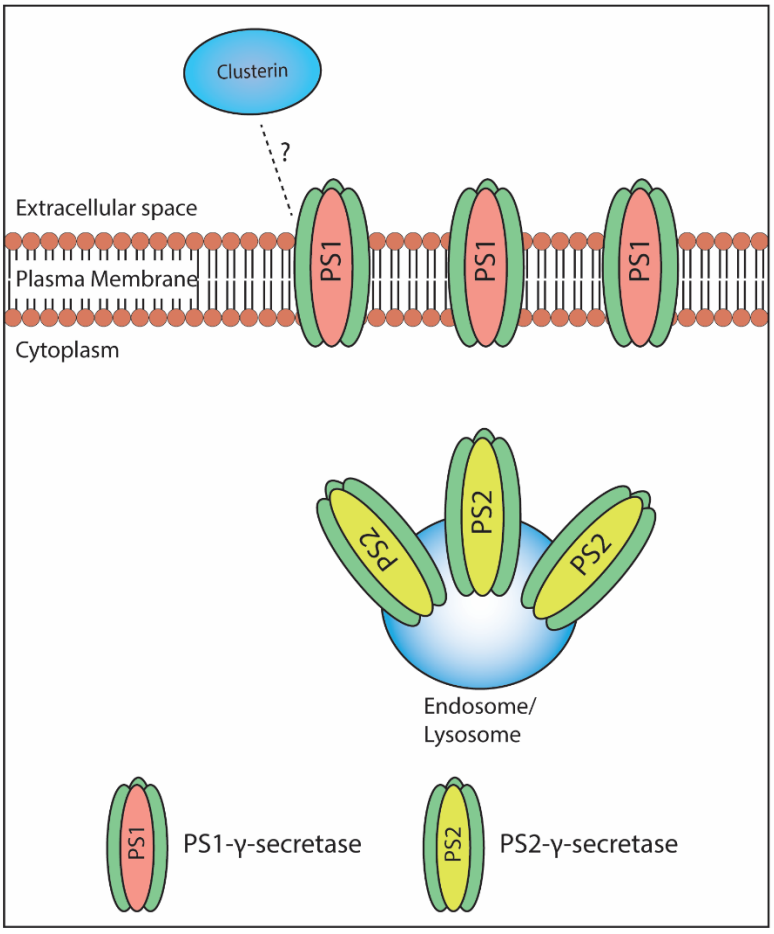

Figure 8: Schematic diagram depicting PS1- vs PS2-γ-secretase subcellular localization. PS1-γ-secretase is mainly localized on the plasma membrane whereas PS2-γ-secretase is localized to endosomes and lysosomes. Clusterin is an extracellular protein and has the potential to interact with PS1-γ-secretase and not PS2-γ-secretase.

In addition to using wild-type SH-SY5Y cells, I also performed the same experiment in BACE1-stably overexpressing cells. In this experiment there was no significant increase in Aβ40 or Aβ42 levels, which was observed as a result of PS1 knockdown in the previous wild-type cell experiment. There was a significant decrease in Aβ34 levels identified as a

result of PS2 knockdown but again, no significant interaction factor between clusterin and either PS1 or PS2 knockdown was detected. From this result, clusterin does not appear to be affecting the PS1- or PS2-catalysed production of Aβ.

Together, the data suggest (1) that clusterin is involved in determining the relative abundance of Aβ peptides in the extracellular space and (2) that neither BACE1 nor γ-secretase cleavage to produce Aβ peptides is affected by clusterin to mediate this effect. Since I have determined that clusterin is not affecting Aβ production to generate the effect on Aβ levels that I observe, I now hypothesise that clusterin is affecting Aβ peptide levels through direct interaction with Aβs in the extracellular space and preventing their degradation. Further experiments to test this hypothesis would be to perform an immunoprecipitation with an anti-clusterin antibody and to probe these samples for Aβ40 or Aβ42. This can be done by SDS-PAGE/Western blot or by mass spectrometry analysis of the immunoprecipitated protein (IP-MS). Recombinantly produced clusterin could be used in combination with synthetic Aβ peptides to assess clusterin's affinity for different Aβs by using surface plasmon resonance (SPR) analysis. Clusterin immobilized on sensor chips could have different Aβ species flowed over the immobilized protein to determine affinity values for clusterin's interaction with different Aβ species and compared with the magnitude of clusterin's effect on these same Aβ peptides. Based on our data, I expect that clusterin will have the highest affinity for Aβ40 followed by Aβ42 and have the lowest affinity for Aβ34.

5. Outlook

The research performed for this thesis has identified a novel role of clusterin in influencing the relative abundance of Aβ peptides. My thesis has advanced our understanding about clusterin and its impact on APP processing which may ultimately help to explain how clusterin contributes to AD pathology. The results obtained in this research project indicate that clusterin likely affects the relative abundance of Aβ peptides in the extracellular space through its modulation of amyloid degradation as opposed to production. Since clusterin is up-regulated in AD, future studies are needed to determine the precise mechanism by which clusterin can increase Aβ peptide levels. If the negative impact of clusterin on amyloid degradation and clearance can be circumvented, this provides a new therapeutic strategy to combat the pathogenesis of AD.

6. Materials

6.1 Buffers

- TAE buffer: 0.4 M Tris, 11.42% acetic acid, EDTA 0.5 M, pH 8
- Luria-Bertani (LB) medium: 1% w/v tryptone, 0.5 % w/v yeast extract, 1 % w/v NaCl
- Phosphate buffered saline (PBS): 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, and a pH of 7.4
- ELISA PBS-T: 1.1 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, 13.7 mM NaCl, (pH 7.4), 0.1% Tween-20
- Whole cell extract (WCE) buffer: 25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.1% Triton-X-100, 0.5 mM dithiothreiol, 4 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete Protease Inhibitor Cocktail (Roche)
- 5x SDS sample buffer: 10% sodium dodecyl sulfate (SDS), 50% glycerol, 25% β-mercaptoethanol, 0.25% bromophenol blue, 0.25M tris H-Cl, pH 6.8
- Tris-glycine running buffer: 25 mM Tris, 190 mM glycine, 0.1% SDS
- MES running buffer: 50mM MES, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH7.3
- 10% ethanol transfer buffer: 25 mM Tris, 200 mM glycine, 10% v/v ethanol
- 1x Ponceau S dye: 0.02% (w/v) Ponceau S, 18.4M trichloroacetic acid, 13.8M sulfosalicylic acid, or 2 g PonceauS**,** 30 g Trichloroacetic acid**,** 30 g Sulfosalicylic acid; for 100ml; working solution: 1:100 in milliQ water
- ECL: (reagent A: 50 mg Luminol in 200 ml, 0.1 M Tris/ HCl, pH 8.6; reagent B: 22 mg p-hydroxy coumaric acid in 20 ml DMSO) Mix 1 part reagent B with 10 parts reagent A, and 0.3µl of 30% H_2O_2 (per ml of reagent A).
- ELISA carbonate buffer: 100 mM sodium carbonate, pH 9.6
- ELISA sample buffer: (90% 11 mM NaH_2PO_4, 85 mM Na_2HPO_4, 137 mM NaCl, (pH 7.4), 0.5% Tween-20, 1.5% BSA, 0.01% Thimerosal, and 10% SeaBlock blocking buffer (Thermo Fisher Scientific Inc.)
- ELISA Mono-HRP buffer: 11 mM NaH_2PO_4, 85 mM Na_2HPO_4, 137 mM NaCl, (pH 7.4), 0.05% Tween-20, 6% PEG

- ELISA Poly-HRP buffer: 1.1 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, 13.7 mM NaCl, (pH 7.4), 0.1% Tween-20, 5% BSA

6.2 Plasmids

Plasmid Name	Plasmid Vector	Insert sequence	Source
pcDNA-Mock	pcDNA3.1	None	Addgene
pcDNA-Clu	pcDNA3.1	Full length clusterin (449 amino acids)	James Eng (Multhaup Laboratory)
pRcCMV-mock	pRcCMV	None	Mark Wilson (University of Wollongong)
pRcCMV-Clu	pRcCMV	Full length clusterin (449 amino acids)	Mark Wilson (University of Wollongong)
Myc-APP-pcDNA	pcDNA3.1	Myc-APP695	Shireen Hossain (Multhaup Laboratory)

6.3 siRNA

siGENOME Non-Targeting siRNA Pool #1 (D-001206-13-05), SMARTpool siGENOME Presenilin 1 (M-004998), and Presenilin 2 (M-006018) were used for control, Presenilin 1 and Presenilin 2 knockdown, respectively.

6.4 Primary antibodies

Antibody Name	Antibody Recognition	Host Species	Dilution factor	Source
G7	Clusterin	Mouse	1: 5000 in PBS	Mark Wilson (University of Wollongong)
WO-2	APP	Mouse	1:5000 in PBS	Multhaup Laboratory
D10E5	BACE1	Rabbit	1:2000 in PBS-T	Abcam
Anti-PS1 (APS18)	Presenilin 1	Rabbit	1:10000 in TBS	Thermo Fischer Scientific Inc.
Anti-PS2 (ab51249)	Presenilin 2	Rabbit	1:10000 in PBS-T	Abcam
C4	Actin	Mouse	1:5000 in PBS	Millipore

6.5 Cell-lines

Cell Line Name	Source	Parent Cell Type	Overexpressed Protein	Plasmid Backbone Used	Selection
HEK-293N	Multhaup Lab	None	None	None	None
SH-SY5Y	Multhaup Lab	None	None	None	None
SH-SY5Y-Mock	Filip Liebsch (Multhaup Lab)	SH-SY5Y Cells	Mock Plasmid	pcDNA3.1	Hygromycin
SH-SY5Y-APP	Filip Liebsch (Multhaup Lab)	SH-SY5Y Cells	APP-695 wild-type	pcDNA3.1	Hygromycin
SH-SY5Y-BACE1	Filip Liebsch (Multhaup Lab)	SH-SY5Y Cells	Human BACE1 wild-type	pcDNA3.1	Hygromycin
HEK-Clu	Travis Rilea (Multhaup Lab)	HEK-293N	Human Clu-449 wild type	pRcCMV	Neomycin
Clu-TEV-HIS	James Eng (Multhaup Lab)	HEK-293N	Human Clu-449-TEV-6xHis	pcDNA3.1	Geneticin (G418)

7. Methods

7.1 Subcloning and DNA preparation

The pcDNA3.1 plasmid containing full-length clusterin insert was made using restriction enzyme digestion/ligation cloning. The starting plasmid: pRcCMV-clu, containing the desired clusterin insert was used for restriction enzyme digestion to remove the coding sequence. The pRcCMV plasmid was digested in a double digestion step using BamHI (NEB) and HindIII (NEB), resulting in several DNA fragments. These DNA fragments were separated using a 1% agarose gel in a horizontal electrophoresis system (BioRad) and TAE running buffer (0.4 M Tris, 11.42% acetic acid, EDTA 0.5 M, pH 8). Following separation, the desired band was extracted using a NucleoSpin® Gel and PCR Cleanup Kit (Macherey Nagel). In tandem, pcDNA3.1 was digested with BamHI and HindIII, treated with calf-intestinal-phosphatase (CIP, NEB) and separated on a 1% agarose gel, followed by gel extraction of the lineralized DNA. DNA was visualized using GelRed® Nucleic Acid Gel Stain (Biotium) and imaged using an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare). Extracted insert and vector DNA was ligated using T4 DNA ligase (NEB) according to the manufacturer's protocol.

DNA plasmids were transformed into NEB® 5-alpha Competent *E. coli* (High Efficiency) according to the manufacturer's protocol. For cloning: 10 colonies were picked and grown in 5ml of Luria-Bertani (LB) medium (1% w/v tryptone, 0.5 % w/v yeast extract, 1 % w/v NaCl), containing selection, overnight at 37°C and 200rpm. The following day 1.5ml of the liquid culture was used for DNA extraction and purification using a NucleoSpin® Plasmid Transfection-grade Kit (Macherey Nagel). Purified plasmid DNA was sent for sanger sequencing at the McGill University and Génome Québec Innovation Centre (Montreal/Canada) prior to midi-preparations.

Once sequences were confirmed, the selected plasmids were amplified in the same manner as described above, using 200ml of LB medium, for midi preps. Batch quantity of plasmid DNA was extracted using a NucleoBond® Xtra Midi / Maxi Kit (Macherey Nagel) following the manufacturer's protocol. Purified DNA was re-dissolved in nuclease-free water and normalized to a concentration of 1mg/ml and stored at -20°C until used.

7.2 Cell Culture and transfections

All cells were cultured on 10cm dishes (Fisher) at 37°C and 5% CO_2. For all transient transfection experiments, cells were seeded on 6-well plates at a density of 300,000 cells per well.

7.2.1 HEK-293 cell seeding

For Human Embryonic Kidney (HEK-293N) cells were cultured in DMEM (High glucose (4.5g/l), 10% fetal bovine serum (FBS), 2mM glutamine, 1 mM pyruvate). For all transient transfection experiments, cells were seeded on 6-well plates. Prior to seeding, 6-well plates were coated with poly-D lysine (Sigma). This was done by diluting poly-D lysine stock solution (Sigma) 1:1000 in sterile water, adding 1.5ml of the solution to each well of a 6-well plate and leaving covered for 1h at room temperature. The solution was as aspirated off and each well was washed with 2ml of fresh sterile water and aspirated. Finally, the plates were left to dry at room temperature for 1h.

Seeding cells was performed by aspirating the culturing medium, washing with 5ml of sterile PBS, the PBS was aspirated and 1ml of Trypsin/EDTA (MultiCell) was added and placed at 37°C for 2min. 9ml of fresh medium was added to the trypsinized cells and the cell suspension was transferred to a sterile tube. Cells were diluted to the appropriate level and seeded onto the poly-D lysine coated plates.

7.2.2 HEK-293 cell transient transfection

HEK-293N cells were transiently transfected using polyethylenimine (PEI, Sigma-Aldrich) 24h after initial seeding. PEI was mixed at a 2:1 ratio to plasmid DNA in OptiMEM (Gibco), mixed by pipetting up and down several times and left at room temperature for 30min. This solution was then diluted in fresh medium at a 1:2 ratio of transfection reagent to fresh media, then added to cells. Generally, 2µg of plasmid DNA, 4µl of PEI were added to 400µl of OptiMEM for each well of a 6-well plate. Cells were left to transfect for 18h then after which the transfection medium was aspirated off and replaced with fresh medium. The transfected cells were now left to condition the medium for 24h before harvesting.

7.2.3 SH-SY5Y cell seeding

SH-HY5Y cells were cultured in DMEM-F12 (MultiCell), 10% FBS. For all transient transfection experiments, SH-SY5Y cells were seeded at a density of 300,000 cells/well in a 6-well plate. The protocol for seeding SH-SY5Y cells was the same as with HEK-293 cells, with the exception that the 6-well plates were not coated with poly-D-lysine prior to seeding.

7.2.4 SH-SY5Y transfections

SH-SY5Y cells were transfected 24h after the cells were seeded. SH-SY5Y cells were transiently transfected using FuGene® HD Transfection Reagent (Promega). For each well, 155µl of OptiMEM was mixed with 3.3µg of DNA plasmid and 10µl of transfection reagent (pre-warmed to 24°C as per the manufacturer's instructions). This solution was gently mixed and incubated at 24°C for 10min. 150µl of the transfection solution was added to each well of a 6-well plate, on top of 2ml of fresh medium. Cells were left in transfection reagent for 24h, then the media was changed and conditioned for an additional 24h before harvesting.

7.2.5 Simultaneous siRNA knockdown and transient transfection in SH-SY5Y cells

Cells were seeded just as in the previous SH-SY5Y experiment. Lipofectamine 2000 Transfection Reagent (Thermo Fischer Scientific Inc.) was used for the simultaneous transfection with plasmid and siRNA treatment. For each well of a 6-well plate, two solutions are required. Solution A: Mix 8µl of Lipofectamine 2000 with 200µl of OptiMEM, Solution B: Mix 1.2µg of plasmid DNA with 15pmol of siRNA and 180µl of OptiMEM. Mix 180µl of Solution A with 180µl of solution B and incubate at 24°C for 5min. Add 300µl of the final transfection mixture to each well, on top of 2ml of fresh cell medium. Cells were allowed to condition the media for 72h before harvesting.

7.3 Harvesting cell media and lysates

Conditioned cell media was collected in 1.5ml tubes, centrifuged at 450rcf, 4°C for 15min then transferred to fresh tubes. Cells were lysed by first washing with cold PBS then adding Whole Cell Extract Buffer (WCE buffer, 25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.1% Triton-X-100, 0.5 mM

dithiothreiol, 4 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete Protease Inhibitor Cocktail (Roche)) and lysing for 1h at 4°C. Cell lysates were then transferred to 1.5ml tubes and centrifuged at 13,000 rcf, 4°C for 15min and the supernatants were transferred to fresh 1.5ml tubes.

7.4 SDS-PAGE and Western blot

7.4.1 SDS-PAGE

Samples were prepared by mixing with 5x SDS sample buffer (10% sodium dodecyl sulfate (SDS), 50% glycerol, 25% β-mercaptoethanol, 0.25% bromophenol blue, 0.25M tris H-Cl, pH 6.8) and boiling at 95°C for 5min. Prepared samples were run on either 10% SDS-PAGE (Bio-Rad) or 4-12% bis-tris (Invitrogen) gels in an electrophoresis chamber using tris-glycine running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) or MES running buffer (50mM MES, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH7.3). Gels were transferred to 0.45μm nitrocellulose membranes (GE) using a wet transfer chamber (BioRad) containing 10% ethanol transfer buffer (25 mM Tris, 200 mM glycine, 10% v/v ethanol).

7.4.2 Western blot

After transfer, membranes were rinsed with distilled water 3-4 times to remove residual transfer buffer, then stained with 1x Ponceau S dye (0.02% (w/v) Ponceau S, 18.4M trichloroacetic acid, 13.8M sulfosalicylic acid) Membranes were blocked for 1-3h at 25°C with shaking in 5% (w/v) skim milk in buffer, either PBS, PBS-T, TBS, TBS-T, depending on antibody compatibility (See materials section 6.4 for list of antibodies and respective buffers). After blocking, membranes were washes with their respective buffer 3-4 times for 5min each time, at 25°C with shaking. Membranes were incubated with primary antibodies overnight at 4°C with shaking. After incubation with primary antibody, membranes were rinsed with their respective buffer 3 times for 10min each time at 25°C with shaking. Membranes were then incubated with secondary antibody (either goat-anti-mouse-HRP or goat-anti-rabbit-HRP, Promega), diluted 1:10,000 in 5% skim milk, for 1h at 25°C with shaking. The secondary antibody was washed off by washing with buffer 3 times for 10 min each time, the same as after incubation with primary antibody.

Membranes were developed using enhanced chemiluminescence (ECL) by mixing 2ml ECL reagent A (50 mg Luminol in 200 ml, 0.1 M Tris/ HCl, pH 8.6) with 200µl ECL reagent B (22 mg p-hydroxy coumaric acid in 20 ml DMSO) and 0.6µl of 30% H_2O_2 for each membrane, and imaged on either an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare) or an Amersham Imager 600 (GE Healthcare).

7.5 Sandwich-bases Enzyme-linked Immunosorbent Assay (ELISA)

96-well ELISA plates (Thermo Fisher Scientific) were coated with neo-epitope specific antibodies against Aβ34, Aβ40, and Aβ42 by diluting in carbonate buffer (100 mM sodium carbonate, pH 9.6), and incubating in the plate overnight at 4°C with gentle shaking. After coating, plates were washed using ELISA PBS-T (1.1 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, 13.7 mM NaCl, (pH 7.4), 0.1% Tween-20) then blocked with StabilCoat® Immunoassay Stabilizer (SurModics Inc). WO2-biotin detection antibody was diluted in ELISA sample buffer (90% 11 mM NaH_2PO_4, 85 mM Na_2HPO_4, 137 mM NaCl, (pH 7.4), 0.5% Tween-20, 1.5% BSA, 0.01% Thimerosal, and 10% SeaBlock blocking buffer (Thermo Fisher Scientific Inc.)) and 50µl was added to each well followed by 50µl of cell culture supernatants sample. Plates were covered and incubated overnight at 4°C with gentle shaking. Plates were washed with ELISA PBS-T followed by addition of Mono-HRP-conjugated streptavidin (SA-Mono-HRP, Pierce) for Aβ40 or poly-HRP-conjugated streptavidin (SA-Poly-HRP, Pierce) for Aβ34 and Aβ42. SA-Mono-HPR was diluted in Mono-HRP buffer (11 mM NaH_2PO_4, 85 mM Na_2HPO_4, 137 mM NaCl, (pH 7.4), 0.05% Tween-20, 6% PEG) and SA-Poly-HRP in Poly HRP buffer (1.1 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, 13.7 mM NaCl, (pH 7.4), 0.1% Tween-20, 5% BSA), added to plates and incubated at room temperature for 1 hour with gentle shaking. Plates were then washed with ELISA PBS-T and room temperature 1-Step™ Ultra TMB-ELISA substrate (Thermo Fisher Scientific Inc.) was added to each well and developed in the dark for a maximum of 30min. The reaction was quenched by adding 1M H_2SO_4 then the samples were read on a Synergy H1 plate reader (BioTek Instruments Inc) by measuring the absorbance at 450nm and 630nm as a reference.

7.6 Immunoprecipitation

Protein G sepharose beads (GE Healthcare) were first washed several times with PBS by adding an excess amount of PBS to the beads, inverting several times to resuspend all beads, then centrifuging at 400rcf, 4°C, for 15min followed by careful aspiration of the supernatant and resuspension of the beads. For the final resuspension of the beads, an equal volume of PBS to beads was used to create a 1:1 slurry of PBS/beads. Capture antibody was then added to the PBS/beads slurry to make a master mix; each sample to be tested required using a ratio of 5µl of antibody to 30µl of PBS/beads. 35µl of master mix was then added to 200-500µl of sample, 20µl of 50x cOmplete Protease Inhibitor Cocktail (Roche), and PBS to have a final volume of 1000µl. Samples were then incubated overnight at 4°C with end-over-end mixing. Beads were pelleted by centrifuging for 5min at 400rcf, 4°C. Supernatant was removed and beads were washed with excess PBS three times by adding cold PBS to the beads, inverting several times to resuspend, centrifuging for 5min at 400rcf, 4°C and aspirating the supernatant. This washing procedure was then repeated using 100mM ammonium acetate (pH 7.4). For the final wash, all the ammonium acetate was removed by aspirating with a 30G ½ needle. Next 350µl of 50% acetic acid was added to each tube and inverted to resuspend the beads. Samples were left at room temperature for 10min then centrifuged at 400rcf for 5min to pellet the beads. 300µl of the supernatant was transferred to a fresh tube and 350µl of fresh 50% acetic acid was added to the beads again and the samples were allowed to sit at room temperature for an additional 10min before centrifuging at 400rcf for 5min. This time 350µl of the supernatant was added to the previous tube containing 300µl of the first elution (final volume of 650µl). Samples were then speed vacuumed, using a Thermo Scientific™ Savant™ SPD131DDA SpeedVac™, for 2.5 hours at ramp setting 5 and at 45℃/hour. Once speed vacuumed, the samples were stored at -80°C until analyzed by western blot or mass spectrometry.

7.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6, employing Student's T-tests, one-way Anova combined with Dunnett's test, and two-way Anova without repeated

measures. For all experiments, technical replicates were averaged to determine the average value for each biological replicate. These calculated average biological replicates were then used as individual data points for statistical analysis. Therefore, the n-number of each experiment corresponds to the number of biological replicates and, by extension, the number of data points used for statistical analysis.

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10. Point to point reply to reviewer comments

Point 1: *The author should mention the occurrence of vascular dementia and how its incidence in the general population compares to Alzheimer's disease.*

Reply 1: In light of this oversight, an addition was made to Section 2.1 (page 7, line 12) to introduce vascular dementia and its relationship to Alzheimer disease. In addition, prevalence rates of both Alzheimer disease and vascular dementia are presented and compared.

Point 2: *The author describes amyloidogenic and non-amyloidogenic processing. What factors determine which pathway dominates in a cell? Does it change with age and/or in different disease conditions? Some commentary on this important issue is needed.*

Reply 2: To further clarify the differences in amyloidogenic vs. non-amyloidogenic processing, a paragraph was added to Section 2.1.2 (page 8, line 27) describing the contributing factors to regulating whether the substrate protein (APP) enters the amyloidogenic or non-amyloidogenic processing pathway. Briefly, these factors include substrate localization and trafficking, and relative enzyme activity levels. To address the second half of this point, a brief review of the literature regarding changes to APP trafficking and enzyme activity in Alzheimer disease was provided and commentary about how these changes could be contributing to Alzheimer disease development was given.

Point 3: *Page 7, Typographical errors; "Amyloid plaques ~~ARE~~ have been found in post-mortem brains of the majority of patients diagnosed with AD [6]".*

Reply 3: The unnecessary word was deleted (page 7, line 18).

Point 4: *The author states "In their 2019 publication (i.e. the Multhaup lab), measuring Aβ peptides related to both amyloid production (i.e. Aβ40, Aβ42) and degradation/clearance (i.e. Aβ34) provided novel insights about the clinical staging of AD". But what were these novel insights? Some expansion on this point would be welcome.*

Reply 4: Additional details regarding the results of this publication were given, as an addition to the final paragraph of Section 2.1.2 (page 10, line 1), and an explanation of their significance for Alzheimer disease diagnosis was provided.

Point 5: *The author makes a compelling argument to why Aβ peptide levels are an important mediator of Alzheimer's pathology. But what about clusterin? According to the Allen brain atlas, clusterin is expressed throughout the brain whereas the author argues that there may be regional- and cell-specific expression of clusterin. This point could be emphasized by making a figure to highlight this point. Does the regional- and cell-specific expression of clusterin help us understand why the onset of Alzheimer's disease often begins in the neocortex? How does clusterin fit into 3 this? Or does the author feel that clusterin plays a role only once the amyloidogenic processing pathway starts to dominate in cells.*

Reply 5: Clarification of the regional differences in clusterin protein vs. mRNA expression were added to Section 2.2.2 (page 15, line 13) to give more detail regarding these regional differences. Further commentary on clusterin's potential involvement and its most likely contribution to Alzheimer disease, based on current evidence, is given in an additional paragraph added to Section 2.2.4 (page 18, line 16).